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OKLAHOMA MEDICAL RESEARCH FOUNDATION [/];
O. TANG, Jordan, J., N. [/]; O. LIN, Xinli [/]; O. KOELSCH,
Gerald [/]; O. PABST, Patrea, L. ; O.

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(54) Title: CATALYTICALLY ACTIVE RECOMBINANT MEMAP SIN AND METHODS OF USE THEREOF

(54) Titre: MEMAPSINE RECOMBINANTE CATALYTIQUEMENT ACTIVE ET PROCEDES D'UTILISATION

(57) Abstract

Methods for the production of purified, catalytically active, recombinant memapsin (2) have been developed. The substrate and subsite specificity of the catalytically active enzyme have been determined. The substrate and subsite specificity information was used to design substrate analogs of the natural memapsin (2) substrate that can inhibit the function of memapsin (2). The substrate analogs are based on peptide sequences, shown to be related to the natural peptide substrates for memapsin (2). The substrate analogs contain at least one analog of an amide bond which is not capable of being cleaved by memapsin (2). Processes for the synthesis of two substrate analogues including isosteres at the sites of the critical amino acid residues were developed and the substrate analogues, OMR99-1 and OM99-2, were synthesized. OM99-2 is based on an octapeptide Glu-Val-Asn-Leu-Ala-Ala-Glu-Phe (SEQ ID NO:28) with the Leu-Ala peptide bond substituted by a transition-state isostere hydroxyethylene group (Fig. 1). The inhibition constant of OM99-2 is 1.6×10^9 M against recombinant pro-memapsin 2. Crystallography of memapsin 2 bound to this inhibitor was used to determine the three dimensional structure of the protein, as well as the importance of the various residues in binding. This information can be used by those skilled in the art to design new inhibitors, using commercially available software programs and techniques familiar to those in organic chemistry and enzymology, to design new inhibitors to memapsin (2), useful in diagnostics and for the treatment and/or prevention of Alzheimer's disease.

(57) Abrégé

L'invention concerne des procédés de production d'une memapsine (2) recombinante purifiée catalytiquement active. La spécificité du substrat et du site secondaire de l'enzyme catalytiquement active a été déterminée. L'information relative à la spécificité du substrat et du site secondaire a été utilisée pour mettre au point des analogues du substrat de la memapsine (2) naturelle pouvant inhiber la fonction de la memapsine (2). Les analogues du substrat sont basés sur des séquences peptidiques dont il est démontré qu'elles sont apparentées aux substrats peptidiques naturels destinés à la memapsine (2). Les analogues du substrat contiennent au moins un analogue d'une liaison amine qui ne peut être clivée par la memapsine (2). Des procédés de synthèse de deux analogues du substrat incluant des isostères aux sites des résidus critiques d'acide aminé ont été mis au point et les analogues du substrat, OMR99-1 et OM99-2, ont été synthétisés. OM99-2 est fondé sur un octapeptide Glu-Val-Asn-Leu-Ala-Ala-Glu-Phe (SEQ ID NO:28) dont la liaison peptidique Leu-Ala a été substituée par un groupe hydroxyéthylène comportant un isostère en état de transition (Fig. 1). La constante d'inhibition d'OM99-2 est $1,6 \times 10^{-9}$ M contre la pro-memapsine (2) recombinante. La cristallographie de la memapsine (2) liée à cet inhibiteur a été utilisée pour déterminer la structure tridimensionnelle de la protéine, ainsi que l'importance des divers résidus dans la liaison. Cette information peut être utilisée par les spécialistes en la matière pour mettre au point de nouveaux inhibiteurs avec des logiciels du commerce et par des techniques connues des spécialistes de la chimie organique ou de l'enzymologie. Ces spécialistes peuvent ainsi mettre au point de nouveaux inhibiteurs de la memapsine (2) à des fins de diagnostic ou pour le traitement et/ou la prévention de la maladie d'Alzheimer.

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- (71) Applicant: OKLAHOMA MEDICAL RESEARCH FOUNDATION [US/US]; 825 N.E. Thirteenth Street, Oklahoma City, OK 73104 (US).
- (72) Inventors: TANG, Jordan, J., N.; 1204 Leawood Drive, Edmond, OK 73003 (US). LIN, Xinli; 1201 Charlton, Edmond, OK 73003 (US). KOELSCH, Gerald; 11516 Roxboro Avenue, Oklahoma City, OK 73162 (US).
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(54) Title: CATALYTICALLY ACTIVE RECOMBINANT MEMAPSPIN AND METHODS OF USE THEREOF

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(57) Abstract: Methods for the production of purified, catalytically active, recombinant memapsin (2) have been developed. The substrate and subsite specificity of the catalytically active enzyme have been determined. The substrate and subsite specificity information was used to design substrate analogs of the natural memapsin (2) substrate that can inhibit the function of memapsin (2). The substrate analogs are based on peptide sequences, shown to be related to the natural peptide substrates for memapsin (2). The substrate analogs contain at least one analog of an amide bond which is not capable of being cleaved by memapsin (2). Processes for the synthesis of two substrate analogues including isosteres at the sites of the critical amino acid residues were developed and the substrate analogues, OMR99-1 and OMR99-2, were synthesized. OMR99-2 is based on an octapeptide Glu-Val-Asn-Leu-Ala-Ala-Glu-Phe (SEQ ID NO:28) with the Leu-Ala peptide bond substituted by a transition-state isostere hydroxyethylene group (Fig. 1). The inhibition constant of OMR99-2 is 1.6×10^9 M against recombinant pro-memapsin 2. Crystallography of memapsin 2 bound to this inhibitor was used to determine the three dimensional structure of the protein, as well as the importance of the various residues in binding. This information can be used by those skilled in the art to design new inhibitors, using commercially available software programs and techniques familiar to those in organic chemistry and enzymology, to design new inhibitors to memapsin (2), useful in diagnostics and for the treatment and/or prevention of Alzheimer's disease.

Description

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CATALYTICALLY ACTIVE RECOMBINANT MEMAP SIN AND METHODS OF USE THEREOF

Background of the Invention

This invention is in the area of the expression of catalytically active Memapsin 2 (beta-secretase) and use thereof in the design and screening of specific inhibitors which are useful in the treatment and/or prevention of Alzheimer's Disease.

Alzheimer's disease (AD) is a degenerative disorder of the brain first described by Alois Alzheimer in 1907 after examining one of his patients who suffered drastic reduction in cognitive abilities and had generalized dementia (*The early story of Alzheimer's Disease*, edited by Bick *et al.* (Raven Press, New York 1987)). It is the leading cause of dementia in elderly persons. AD patients have increased problems with memory loss and intellectual functions which progress to the point where they cannot function as normal individuals. With the loss of intellectual skills the patients exhibit personality changes, socially inappropriate actions and schizophrenia (*A Guide to the Understanding of Alzheimer's Disease and Related Disorders*, edited by Jorm (New York University Press, New York 1987). AD is devastating for both victims and their families, for there is no effective palliative or preventive treatment for the inevitable neurodegeneration.

AD is associated with neuritic plaques measuring up to 200 μ m in diameter in the cortex, hippocampus, subiculum, hippocampal gyrus, and amygdala. One of the principal constituents of neuritic plaques is amyloid, which is stained by Congo Red (Fisher (1983); Kelly Microbiol. Sci. 1(9):214-219 (1984)). Amyloid plaques stained by Congo Red are extracellular, pink or rust-colored in bright field, and birefringent in polarized light. The plaques are composed of polypeptide fibrils and are often present around blood vessels, reducing blood supply to various neurons in the brain.

Various factors such as genetic predisposition, infectious agents, toxins, metals, and head trauma have all been suggested as possible mechanisms of AD neuropathy. Available evidence strongly indicates that there are distinct types of genetic predispositions for AD. First, molecular

5 analysis has provided evidence for mutations in the amyloid precursor
protein (APP) gene in certain AD-stricken families (Goate *et al.* *Nature*
349:704-706 (1991); Murrell *et al.* *Science* 254:97-99 (1991); Chartier-
10 Harlin *et al.* *Nature* 353:844-846 (1991); Mullan *et al.*, *Nature Genet.* 1:345-
347 (1992)). Additional genes for dominant forms of early onset AD reside
on chromosome 14 and chromosome 1 (Rogaev *et al.*, *Nature* 376:775-778
(1995); Levy-Lahad *et al.*, *Science* 269:973-977 (1995); Sherrington *et al.*,
15 *Nature* 375:754-760 (1995)). Another loci associated with AD resides on
chromosome 19 and encodes a variant form of apolipoprotein E (Corder,
Science 261:921-923 (1993)).

20 Amyloid plaques are abundantly present in AD patients and in
Down's Syndrome individuals surviving to the age of 40. The
overexpression of APP in Down's Syndrome is recognized as a possible
cause of the development of AD in Down's patients over thirty years of age
25 (Rumble *et al.*, *New England J. Med.* 320:1446-1452 (1989); Mann *et al.*,
Neurobiol. Aging 10:397-399 (1989)). The plaques are also present in the
normal aging brain, although at a lower number. These plaques are made up
primarily of the amyloid β peptide ($A\beta$; sometimes also referred to in the
30 literature as β -amyloid peptide or β peptide) (Glennner and Wong, *Biochem.*
Biophys. Res. Comm. 120:885-890 (1984)), which is also the primary protein
constituent in cerebrovascular amyloid deposits. The amyloid is a
35 filamentous material that is arranged in beta-pleated sheets. $A\beta$ is a
hydrophobic peptide comprising up to 43 amino acids.

The determination of its amino acid sequence led to the cloning of the
APP cDNA (Kang *et al.*, *Nature* 325:733-735 (1987); Goldgaber *et al.*,
40 *Science* 235:877-880 (1987); Robakis *et al.*, *Proc. Natl. Acad. Sci.* 84:4190-
4194 (1987); Tanzi *et al.*, *Nature* 331:528-530 (1988)) and genomic APP
DNA (Lemaire *et al.*, *Nucl. Acids Res.* 17:517-522 (1989); Yoshikai *et al.*,
Gene 87, 257-263 (1990)). A number of forms of APP cDNA have been
45 identified, including the three most abundant forms, APP695, APP751, and
APP770. These forms arise from a single precursor RNA by alternate
splicing. The gene spans more than 175 kb with 18 exons (Yoshikai *et al.*

(1990)). APP contains an extracellular domain, a transmembrane region and a cytoplasmic domain. A β consists of up to 28 amino acids just outside the hydrophobic transmembrane domain and up to 15 residues of this transmembrane domain. A β is normally found in brain and other tissues such as heart, kidney and spleen. However, A β deposits are usually found in abundance only in the brain.

Van Broeckhoven *et al.*, *Science* 248:1120-1122 (1990), have demonstrated that the APP gene is tightly linked to hereditary cerebral hemorrhage with amyloidosis (HCHWA-D) in two Dutch families. This was confirmed by the finding of a point mutation in the APP coding region in two Dutch patients (Levy *et al.*, *Science* 248:1124-1128 (1990)). The mutation substituted a glutamine for glutamic acid at position 22 of the A β (position 618 of APP695, or position 693 of APP770). In addition, certain families are genetically predisposed to Alzheimer's disease, a condition referred to as familial Alzheimer's disease (FAD), through mutations resulting in an amino acid replacement at position 717 of the full length protein (Goate *et al.* (1991); Murrell *et al.* (1991); Chartier-Harlin *et al.* (1991)). These mutations co-segregate with the disease within the families and are absent in families with late-onset AD. This mutation at amino acid 717 increases the production of the A β ₁₋₄₂ form of A β from APP (Suzuki *et al.*, *Science* 264:1336-1340 (1994)). Another mutant form contains a change in amino acids at positions 670 and 671 of the full length protein (Mullan *et al.* (1992)). This mutation to amino acids 670 and 671 increases the production of total A β from APP (Citron *et al.*, *Nature* 360:622-674 (1992)).

APP is processed *in vivo* at three sites. The evidence suggests that cleavage at the β -secretase site by a membrane associated metalloprotease is a physiological event. This site is located in APP 12 residues away from the luminal surface of the plasma membrane. Cleavage of the β -secretase site (28 residues from the plasma membrane's luminal surface) and the β -secretase site (in the transmembrane region) results in the 40/42-residue β -amyloid peptide (A β), whose elevated production and accumulation in the brain are the central events in the pathogenesis of Alzheimer's disease (for

5 review, see Selkoe, D.J. *Nature* 399:23-31 (1999)). Presenilin 1, another
membrane protein found in human brain, controls the hydrolysis at the APP (
10 β -secretase site and has been postulated to be itself the responsible protease
(Wolfe, M.S. et al., *Nature* 398:513-517 (1999)). Presenilin 1 is expressed as
a single chain molecule and its processing by a protease, presenilinase, is
required to prevent it from rapid degradation (Thinakaran, G. et al., *Neuron*
15 17:181-190 (1996) and Podlisny, M.B., et al., *Neurobiol. Dis.* 3:325-37
(1997)). The identity of presenilinase is unknown. The *in vivo* processing of
the β -secretase site is thought to be the rate-limiting step in A β production
(Sinha, S. & Lieberburg, I., *Proc. Natl. Acad. Sci. USA*, 96:11049-11053
(1999)), and is therefore a strong therapeutic target.

20 The design of inhibitors effective in decreasing amyloid plaque
formation is dependent on the identification of the critical enzyme(s) in the
cleavage of APP to yield the 42 amino acid peptide, the A β_{1-42} form of A β .
25 Although several enzymes have been identified, it has not been possible to
produce active enzyme. Without active enzyme, one cannot confirm the
substrate specificity, determine the subsite specificity, nor determine the
kinetics or critical active site residues, all of which are essential for the
30 design of inhibitors.

Memapsin 2 has been shown to be beta-secretase, a key protease
involved in the production in human brain of beta-amyloid peptide from
35 beta-amyloid precursor protein (for review, see Selkoe, D.J. *Nature* 399:23-
31 (1999)). It is now generally accepted that the accumulation of beta-
amyloid peptide in human brain is a major cause for the Alzheimer's
disease. Inhibitors specifically designed for human memapsin 2 should
40 inhibit or decrease the formation of beta-amyloid peptide and the
progression of the Alzheimer's disease.

Memapsin 2 belongs to the aspartic protease family. It is
45 homologous in amino acid sequence to other eukaryotic aspartic proteases
and contains motifs specific to that family. These structural similarities
predict that memapsin 2 and other eukaryotic aspartic proteases share
common catalytic mechanism Davies, D.R., *Annu. Rev. Biophys. Chem.* 19,
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189 (1990). The most successful inhibitors for aspartic proteases are mimics of the transition state of these enzymes. These inhibitors have substrate-like structure with the cleaved planar peptide bond between the carbonyl carbon and the amide nitrogen replaced by two tetrahedral atoms, such as hydroxyethylene [-CH(OH)-CH₂-], which was originally discovered in the structure of pepstatin (Marciniszyn et al., 1976).

However, for clinical use, it is preferable to have small molecule inhibitors which will pass through the blood brain barrier and which can be readily synthesized. It is also desirable that the inhibitors are relatively inexpensive to manufacture and that they can be administered orally. Screening of thousands of compounds for these properties would require an enormous effort. To rationally design memapsin 2 inhibitors for treating Alzheimer's disease, it will be important to know the three-dimensional structure of memapsin 2, especially the binding mode of an inhibitor in the active site of this protease.

It is therefore an object of the present invention to provide purified, recombinant, and active memapsin 2, as well as its substrate and subsite specificity and critical active site residues.

It is a further object of the present invention to provide compositions and methods for synthesis of inhibitors of memapsin 2.

It is a still further object of the present invention to provide compositions that interact with memapsin 2 or its substrate to inhibit cleavage by the memapsin 2 which can cross the blood brain barrier (BBB).

It is therefore an object of the present invention to provide means for rational design and screening of compounds for inhibition of memapsin 2.

Summary of the Invention

Methods for the production of purified, catalytically active, recombinant memapsin 2 have been developed. The substrate and subsite specificity of the catalytically active enzyme have been determined. The active enzyme and assays for catalytic activity are useful in screening libraries for inhibitors of the enzyme.

5 The substrate and subsite specificity information was used to design
substrate analogs of the natural memapsin 2 substrate that can inhibit the
function of memapsin 2. The substrate analogs are based on peptide
10 sequences, shown to be related to the natural peptide substrates for
memapsin 2. The substrate analogs contain at least one analog of an amide
(peptide) bond which is not capable of being cleaved by memapsin 2.
Processes for the synthesis of two substrate analogues including isosteres at
15 the sites of the critical amino acid residues were developed and the substrate
analogues, OMR99-1 and OM99-2, were synthesized. OM99-2 is based on
an octapeptide Glu-Val-Asn-Leu-Ala-Ala-Glu-Phe (SEQ ID NO:28) with
the Leu-Ala peptide bond substituted by a transition-state isostere
20 hydroxyethylene group. The inhibition constant of OM99-2 is 1.6×10^{-9} M
against recombinant pro-memapsin 2. Crystallography of memapsin 2
bound to this inhibitor was used to determine the three dimensional
structure of the protein, as well as the importance of the various residues in
25 binding.

 This information can be used by those skilled in the art to design
new inhibitors, using commercially available software programs and
30 techniques familiar to those in organic chemistry and enzymology, to design
new inhibitors. For example, the side chains of the inhibitors may be
modified to produce stronger interactions (through hydrogen bonding,
hydrophobic interaction, charge interaction and/or van der Waal interaction)
35 in order to increase inhibition potency. Based on this type of information,
the residues with minor interactions may be eliminated from the new
inhibitor design to decrease the molecular weight of the inhibitor. The side
chains with no structural hindrance from the enzyme may be cross-linked to
40 lock in the effective inhibitor conformation. This type of structure also
enables the design of peptide surrogates which may effectively fill the
binding sites of memapsin 2 yet produce better pharmaceutical properties.

45 The examples demonstrate the production of catalytically active
enzyme, design and synthesis of inhibitors, and how the crystal structure was
obtained. The examples thereby demonstrate how the methods and materials

described herein can be used to screen libraries of compounds for other inhibitors, as well as for design of inhibitors. These inhibitors are useful in the prevention and/or treatment of Alzheimer's disease as mediated by the action of the beta secretase memapsin 2, in cleaving APP.

Brief Description of the Drawings

Figure 1 depicts the plasmid construct of vector pET-11a-memapsin 2-T1 and pET-11a-memapsin 2-T2. The T7 promotor, amino acid sequence from the vector (T7 protein) (SEQ ID NO:3), and the beginning and ending of the memapsin 2 T1 and T2 construct are shown. Construct promemapsin 2-T1 was used in the preparation of protein for crystallization and includes residues 1v-15v which are derived from vector pET-11a. Residues 1p-48p are putative pro-peptide. Residues 1-393 correspond to the mature protease domain and C-terminal extension. The residue numbering of memapsin 2 starts at the aligned N-terminal position of pepsin (Figure 3).

Figure 2A is a graph of the initial rate of hydrolysis of synthetic peptide swAPP (see Table 1) by $M2_{pd}$ at different pH. Figure 2B is a graph of the relative k_{cat}/K_m values for steady-state kinetic of hydrolysis of peptide substrates by $M2_{pd}$.

Figures 3A and 3B are the chemical structures of memapsin 2 inhibitors, OM99-2 and OM99-1.

Figure 4A is a graph of the inhibition of recombinant memapsin 2 by OM99-1. Figure 4B is a graph of the inhibition of recombinant memapsin 2 by OM99-2.

Figures 5A-E are photographs of crystals of recombinant memapsin 2-OM99-2 complex.

Figure 6 is a stereo view of crystal structure of memapsin 2 protease domain with bound OM99-2. The polypeptide backbone of memapsin 2 is shown as a ribbon diagram. The N-lobe and C-lobe are blue and yellow, respectively, except the insertion loops (designated A to G, see Figure 2) in the C-lobe are magenta and the C-terminal extension is green. The inhibitor bound between the lobes is shown in red.

Figure 7 is a stereo view of comparison of the three-dimensional structures of memapsin 2 and pepsin. The molecular surface of the former is significantly larger by the insertion of surface loops and helix and the C-terminal extension. Chain tracing of human memapsin 2 is dark blue and is grey for human pepsin. The light blue balls represent identical residues which are topologically equivalent. The disulfide bonds are shown in red for memapsin 2 and orange for pepsin. The C-terminal extension is in green.

Figure 8 is a schematic presentation of interaction between OM99-2 and memapsin 2 protease domain. The S_3' and S_4' subsites are not defined.

Figure 9 is a stereo presentation of interactions between inhibitor OM99-2 (orange) and memapsin 2 (light blue). Nitrogen and oxygen atoms are marked blue and red, respectively. Hydrogen bonds are indicated in yellow dotted lines. Memapsin 2 residues which comprise the binding subsites are included. Residues P_4 , P_3 , P_2 , P_1 and P_1' (defined in Figure 8) of OM99-2 are in an extended conformation. Inhibitor chain turns at residue P_2' which makes a distinct kink at this position. The backbone of P_3' and P_4' directs the inhibitor to exit the active site.

Figure 10 are schematics of the cross linking between P_3 Val and P_1 Leu side chains in the design of new inhibitors for memapsin 2 based on the current crystal structure. R and R' at positions P_2 and P_1' indicate amino acid side chains. Other structural elements of inhibitor are omitted for clarity.

Figure 11 are schematics of the cross linking between P_4 Glu and P_2 Asn side chains in the design of new inhibitors for memapsin 2 based on the current crystal structure. R at position P_3 indicates amino acid side chain. Other structural elements of inhibitor are omitted for clarity.

Figure 12 is a schematic of the design for the side chain at the P_1' subsite for the new memapsin 2 inhibitors based on the current crystal structure. Arrows indicate possible interactions between memapsin 2 and inhibitor. Other structural elements of inhibitor are omitted for clarity.

Figure 13 is a schematic of the design of two six-membered rings in the inhibitor structure by the addition of atoms A and B. The ring formation

5 involves the P₁-Leu side chain the the peptide backbone near P₁, P₂, and P₃.
The new bonds are in dotted lines. A methyl group can be added to the beta-
carbon of P₁-Leu. Other structural elements of inhibitor are omitted for
10 clarity.

Detailed Description of the Invention

I. Preparation of Catalytically Active Recombinant Memapsin 2

Cloning and Expression of Memapsin 2

15 Memapsin 2 was cloned and the nucleotide (SEQ ID NO. 1) and
predicted amino acid (SEQ ID NO. 2) sequences were determined, as
described in Example 1. The cDNA was assembled from the fragments. The
nucleotide and the deduced protein sequence are shown in SEQ ID NOs. 1
20 and 2, respectively. The protein is the same as the aspartic proteinase 2
(ASP2) described in EP 0 855 444 A by SmithKline Beecham
Pharmaceuticals, (published July 29, 1998), and later reported by Sinha, et
al., Nature 402, 537-540 (December 1999) and Vassar, et al., Science 286,
25 735-741 (22 October 1999).

Pro-memapsin 2 is homologous to other human aspartic proteases.
Based on the alignments, Pro-memapsin 2 contains a *pro* region, an aspartic
30 protease region, and a trans-membrane region near the C-terminus. The C-
terminal domain is over 80 residues long. The active enzyme is memapsin 2
and its pro-enzyme is pro-memapsin 2.

Refolding Catalytically Active Enzyme

35 In order to determine the substrate specificity and to design
inhibitors, it is necessary to express catalytically active recombinant enzyme.
No other known proteases contain a transmembrane domain. The presence
of transmembrane domains makes the recombinant expression of these
40 proteins less predictable and more difficult. The transmembrane region often
needs to be removed so that secretion of the protein can take place.
However, the removal of the transmembrane region can often alter the
45 structure and/or function of the protein.

The starting assumption was that the region of memapsin 2 that is
homologous with other aspartic proteases would independently fold in the

5 absence of the transmembrane domain, and would retain protease activity in
the absence of the C-terminal transmembrane region. The transmembrane
region appears to serve as a membrane anchor. Since the active site is not in
10 the transmembrane region and activity does not require membrane
anchoring, memapsin 2 was expressed in *E. coli* in two different lengths,
both without the transmembrane region, and purified, as described in
Example 3. The procedures for the culture of transfected bacteria, induction
15 of synthesis of recombinant proteins and the recovery and washing of
inclusion bodies containing recombinant proteins are essentially as described
by Lin et al., (1994). Refolding was not a simple matter, however. Two
different refolding methods both produced satisfactory results. In both
20 methods, the protein was dissolved in a strong denaturing/reducing solution
such as 8 M urea/100 mM beta-mercaptoethanol. The rate at which the
protein was refolded, and in what solution, was critical to activity. In one
method, the protein is dissolved into 8 M urea/100 mM beta-
25 mercaptoethanol then rapidly diluted into 20 volumes of 20 mM-Tris, pH
9.0, which is then slowly adjusted to pH 8 with 1 M HCl. The refolding
solution was then kept at 4°C for 24 to 48 hours before proceeding with
purification. In the second method, an equal volume of 20 mM Tris, 0.5 mM
30 oxidized/1.25 mM reduced glutathione, pH 9.0 is added to rapidly stirred
pro-memapsin 2 in 8 M urea/10 mM beta-mercaptoethanol. The process is
repeated three more times with 1 hour intervals. The resulting solution is
then dialyzed against sufficient volume of 20 mM Tris base so that the final
35 urea concentration is 0.4 M. The pH of the solution is then slowly adjusted to
8.0 with 1 M HCl.

40 The refolded protein is then further purified by column
chromatography, based on molecular weight exclusion, and/or elution using
a salt gradient, and analyzed by SDS-PAGE analysis under reduced and non-
45 reduced conditions.

II. Substrate Specificity and Enzyme Kinetics of Memapsin 2

Substrate Specificity

The tissue distribution of the memapsin 2 was determined, as described in Example 2. The presence of memapsin 2 (M2) in the brain indicated that it might hydrolyze the β -amyloid precursor protein (APP). As described below, detailed enzymatic and cellular studies demonstrated that M2 fits all the criteria of the β -secretase.

The M2 three-dimensional structure modeled as a type I integral membrane protein. The model suggested that its globular protease unit can hydrolyze a membrane anchored polypeptide at a distance range of 20-30 residues from the membrane surface. As a transmembrane protein of the brain, APP is a potential substrate and its beta-secretase site, located about 28 residues from the plasma membrane surface, is within in the range for M2 proteolysis.

A synthetic peptide derived from this site (SEVKM/DAEFR) (SEQ ID NO:4) was hydrolyzed by M2_{pd} (modified M2 containing amino acids from Ala⁸¹ to Ala³²⁶) at the beta-secretase site (marked by /). A second peptide (SEVNL/DAEFR) (SEQ ID NO:5) derived from the APP beta-secretase site and containing the 'Swedish mutation' (Mullan, M. *et al.*, *Nature Genet.* 2:340-342 (1992)), known to elevate the level of alpha-beta production in cells (Citron, M. *et al.*, *Nature* 260:672-674 (1992)), was hydrolyzed by M2_{pd} with much higher catalytic efficiency. Both substrates were optimally cleaved at pH 4.0. A peptide derived from the processing site of presenilin 1 (SVNM/AEGD) (SEQ ID NO:6) was also cleaved by M2_{pd} with less efficient kinetic parameters. A peptide derived from the APP gamma-secretase site (KGGVVIATVIVK) (SEQ ID NO:7) was not cleaved by M2_{pd}. Pepstatin A inhibited M2_{pd} poorly (IC₅₀ approximately 0.3 mM). The kinetic parameters indicate that both presenilin 1 (k_{cat} , 0.67 s⁻¹; K_m , 15.2 mM; k_{cat}/K_m , 43.8 s⁻¹M⁻¹) and native APP peptides (k_{cat}/K_m , 39.9 s⁻¹M⁻¹) are not as good substrates as the Swedish APP peptide (k_{cat} , 2.45 s⁻¹; K_m , 1 mM; k_{cat}/K_m , 2450 s⁻¹M⁻¹).

5 To determine if M2 possesses an APP beta-secretase function in
mammalian cells, memapsin 2 was transiently expressed in HeLa cells (Lin.
X., et al., *FASEB J.* 7:1070-1080 (1993)), metabolically pulse-labeled with
10 ³⁵S-Met, then immunoprecipitated with anti-APP antibodies for visualization
of APP-generated fragments after SDS-polyacrylamide electrophoresis and
imaging. SDS-PAGE patterns of immuno-precipitated APP N β -fragment
(97 kD band) from the conditioned media (2 h) of pulse-chase experiments
15 showed that APP was cleaved by M2. Controls transfected with APP alone
and co-transfected with APP and M2 with Bafilomycin A1 added were
performed. SDS-PAGE patterns of APP β C-fragment (12 kD) were
immunoprecipitated from the conditioned media of the same experiment as
discussed above. Controls transfected with APP alone; co-transfected with
20 APP and M2; co-transfected with APP and M2 with Bafilomycin A1;
transfections of Swedish APP; and co-transfections of Swedish APP and M2
were performed. SDS-PAGE gels were also run of immuno-precipitated M2
25 (70 kD), M2 transfected cells; untransfected HeLa cells after long time film
exposure; and endogenous M2 from HEK 293 cells. SDS-PAGE patterns of
APP fragments (100 kD betaN-fragment and 95 kD betaN-fragment)
30 recovered from conditioned media after immuno-precipitation using
antibodies specific for different APP regions indicated that memapsin 2
cleaved APP.

35 Cells expressing both APP and M2 produced the 97 kD APP beta N-
fragment (from the N-terminus to the beta-secretase site) in the conditioned
media and the 12 kD betaC-fragment (from the beta-secretase site to the C-
terminus) in the cell lysate. Controls transfected with APP alone produced
40 little detectable betaN-fragment and no beta C-fragment. Bafilomycin A1,
which is known to raise the intra-vesicle pH of lysosomes/endosomes and
has been shown to inhibit APP cleavage by beta-secretase (Knops, J. et al., *J.*
Biol. Chem. 270:2419-2422 (1995)), abolished the production of both APP
45 fragments beta N- and beta C- in co-transfected cells. Cells transfected with
Swedish APP alone did not produce the beta C-fragment band in the cell
lysate but the co-transfection of Swedish APP and M2 did. This Swedish

5 beta C-fragment band is more intense than that of wild-type APP. A 97-kD
beta N-band is also seen in the conditioned media but is about equal intensity
as the wild-type APP transfection.

10 These results indicate that M2 processes the beta-secretase site of
APP in acidic compartments such as the endosomes. To establish the
expression of transfected M2 gene, the pulse-labeled cells were lysed and
immuno-precipitated by anti-M2 antibodies. A 70 kD M2 band was seen in
15 cells transfected with M2 gene, which has the same mobility as the major
band from HEK 293 cells known to express beta-secretase (Citron, M. et al.,
Nature 260:672-674 (1992)). A very faint band of M2 is also seen, after a
long film exposure, in untransfected HeLa cells, indicating a very low level
20 of endogenous M2, which is insufficient to produce betaN- or betaC-
fragments without M2 transfection. Antibody alpha-beta₁₋₁₇, which
specifically recognizes residues 1-17 in alpha-beta peptide, was used to
confirm the correct beta-secretase site cleavage. In cells transfected with
25 APP and M2, both beta N- and beta N-fragments are visible using an
antibody recognizing the N-terminal region of APP present in both
fragments. Antibody Abeta₁₋₁₇ recognize the beta N-fragment produced by
endogenous beta-secretase in the untransfected cells. This antibody was,
30 however, unable to recognize the betaN-fragment known to be present in
cells co-transfected with APP and M2. These observations confirmed that
betaN-fragment is the product of beta-secretase site cut by M2, which
abolished the recognition epitope of alpha-beta₁₋₁₇.

35 The processing of APP by M2 predicts the intracellular colocalization
of the two proteins. HeLa cells co-expressing APP and M2 were stained
with antibodies directed toward APP and M2 and visualized simultaneously
40 by CSLM using a 100x objective. Areas of colocalization appeared in
yellow.

45 Immunodetection observed by confocal microscopy of both APP and
M2 revealed their colocalization in the superimposed scans. The distribution
of both proteins is consistent with their residence in lysosomal/endosomal
compartments.

5 In specificity studies, it was found that M2_{pd} cleaved its *pro* peptide
(2 sites) and the protease portion (2 sites) during a 16 h incubation after
activation (Table 1). Besides the three peptides discussed above, M2_{pd} also
10- cleaved oxidized bovine insulin B chain and a synthetic peptide Nch. Native
proteins were not cleaved by M2_{pd}.

The data indicate that human M2 fulfills all the criteria of a beta-
secretase which cleaves the beta-amyloid precursor protein (APP): (a) M2
15 and APP are both membrane proteins present in human brain and co-localize
in mammalian cells, (b) M2 specifically cleaves the beta-secretase site of
synthetic peptides and of APP in cells, (c) M2 preferentially cleaves the beta-
secretase site from the Swedish over the wild-type APP, and (d) the acidic
20 pH optimum for M2 activity and bafilomycin A1 inhibition of APP
processing by M2 in the cells are consistent with the previous observations
that beta-secretase cleavage occurs in acidic vesicles (Knops, J., et al., *J.*
Biol. Chem. 270:2419-2422 (1995)). The spontaneous appearance of activity
25 of recombinant pro-M2 in an acidic solution suggests that, intracellularly,
this zymogen can by itself generate activity in an acidic vesicle like an
endosome.

30 II. Design and Synthesis of Inhibitors

Design of Substrate Analogs for Memapsin 2.

The five human aspartic proteases have homologous amino acid
35 sequences and have similar three-dimensional structures. There are two
aspartic residues in the active site and each residue is found within the
signature aspartic protease sequence motif, Asp-Thr/Ser-Gly- (SEQ ID
NO:8). There are generally two homologous domains within an aspartic
40 protease and the substrate binding site is positioned between these two
domains, based on the three-dimensional structures. The substrate binding
sites of aspartic proteases generally recognize eight amino acid residues.
There are generally four residues on each side of the amide bond which is
45 cleaved by the aspartic protease.

Typically the side chains of each amino acid are involved in the
specificity of the substrate/aspartic protease interaction. The side chain of

each substrate residue is recognized by regions of the enzyme which are collectively called sub-sites. The generally accepted nomenclature for the protease sub-sites and their corresponding substrate residues are shown below, where the double slash represents the position of bond cleavage.

Protease sub-sites	S4	S3	S2	S1	S1'	S2'	S3'	S4'
Substrate residues	P4	P3	P2	P1	// P1'	P2'	P3'	P4'

While there is a general motif for aspartic protease substrate recognition, each protease has a very different substrate specificity and breadth of specificity. Once the specificity of an aspartic protease is known, inhibitors can be designed based on that specificity, which interact with the aspartic protease in a way that prevents natural substrate from being efficiently cleaved. Some aspartic proteases have specificities which can accommodate many different residues in each of the sub-sites for successful hydrolysis. Pepsin and cathepsin D have this type of specificity and are said to have "broad" substrate specificity. When only a very few residues can be recognized at a sub-site, such as in renin, the aspartic protease is said to have a stringent or narrow specificity.

The information on the specificity of an aspartic protease can be used to design specific inhibitors in which the preferred residues are placed at specific sub-sites and the cleaved peptide bond is replaced by an analog of the transition-state. These analogs are called transition state isosteres. Aspartic proteases cleave amide bonds by a hydrolytic mechanism. This reaction mechanism involves the attack by a hydroxide ion on the β -carbon of the amino acid. Protonation must occur at the other atom attached to the β -carbon through the bond that is to be cleaved. If the β -carbon is insufficiently electrophilic or the atom attached to the bond to be cleaved is insufficiently nucleophilic the bond will not be cleaved by a hydrolytic mechanism. Analogs exist which do not mimic the transition state but which are non-hydrolyzable, but transition state isosteres mimic the transition state specifically and are non-hydrolyzable.

Transition state theory indicates that it is the transition state intermediate of the reaction which the enzyme catalyzes for which the

enzyme has its highest affinity. It is the transition state structure, not the ground state structure, of the substrate which will have the highest affinity for its given enzyme. The transition state for the hydrolysis of an amide bond is tetrahedral while the ground state structure is planar. A typical transition-state isostere of aspartic protease is $-\text{CH}(\text{OH})-\text{CH}_2-$, as was first discovered in pepstatin by Marcinişzyn et al. (1976). The transition-state analogue principles have been successfully applied to inhibitor drugs for human immunodeficiency virus protease, an aspartic protease. Many of these are currently in clinical use. Information on the structure, specificity, and types of inhibitors can be found in Tang, Acid Proteases, Structure, Function and Biology, Adv. in Exptl. Med. Biol. vol. 95 (Plenum Press, NY 1977); Kostka, Aspartic Proteinases and their Inhibitors (Walter de Gruyter, Berlin 1985); Dunn, Structure and Functions of the Aspartic Proteinases, Adv. in Exptl. Med. Biol. 306 (Plenum Press, NY 1991); Takahashi, Aspartic Proteases, Structure, Function, Biology, Biomedical Implications, Adv. in Exptl. Med. Biol. 362 (Plenum Press, NY 1995); and James, Aspartic Proteinases, Retroviral and Cellular Enzymes, Adv. in Exptl. Med. Biol. 436 (Plenum Press, NY 1998)).

Substrate analog compositions are generally of the general formula $X-L_1-P_1-L_2-P_2-L_3-P_3-L_4-P_4-L_5-P_5-L_6-P_6-L_7-P_7-L_8-P_8-L_9-P_9-L_{10}-Y$. The substrate analog compositions are analogs of small peptide molecules. Their basic structure is derived from peptide sequences that were determined through structure/function studies. It is understood that positions represented by P_x represent the substrate specificity position relative to the cleavage site which is represented by an $-L_0-$. The positions of the compositions represented by L_x represent the linking regions between each substrate specificity position, P_x .

In a natural substrate for memapsin 2, a P_x-L_x pair would represent a single amino acid of the peptide which is to be cleaved. In the present general formula, each P_x part of the formula refers to the α -carbon and side chain functional group of each would be amino acid. Thus, the P_x portion of

an P_x-L_x pair for alanine represents $HC-CH_3$. The general formula representing the P_x portion of the general composition is $-R_1CR_3-$.

In general R_1 can be either CH_3 (side chain of alanine), $CH(CH_3)_2$ (side chain of valine), $CH_2CH(CH_3)_2$ (side chain of leucine), $(CH_3)CH(CH_2CH_3)$ (side chain of isoleucine), CH_2 (Indole) (side chain of tryptophan), CH_2 (Benzene) (side chain of phenylalanine), $CH_2CH_2SCH_3$ (side chain of methionine), H (side chain of glycine), CH_2OH (side chain of serine), $CHOHCH_3$ (side chain of threonine), $CH_2(Phenol)$ (side chain of tyrosine), CH_2SH (side chain of cysteine), $CH_2CH_2CONH_2$ (side chain of glutamine), CH_2CONH_2 (side chain of asparagine), $CH_2CH_2CH_2CH_2NH_2$ (side chain of lysine), $CH_2CH_2CH_2NHC(NH)(NH_2)$ (side chain of arginine), CH_2 (Imidazole) (side chain of histidine), CH_2COOH (side chain of aspartic acid), CH_2CH_2COOH (side chain of glutamic acid), and functional natural and non-natural derivatives or synthetic substitutions of these.

It is most preferred that R_3 is a single H . In general, however, R_3 can be alkenyl, alkynyl, alkenyloxy, and alkynyloxy groups that allow binding to memapsin 2. Preferably, alkenyl, alkynyl, alkenyloxy and alkynyloxy groups have from 2 to 40 carbons, and more preferably from 2 to 20 carbons, from 2 to 10 carbons, or from 2 to 3 carbons, and functional natural and non-natural derivatives or synthetic substitutions of these.

The L_x portion of the P_x-L_x pair represents the atoms linking the P_x regions together. In a natural substrate the L_x represents the β -carbon attached to the amino portion of what would be the next amino acid in the chain. Thus, L_x would be represented by $-CO-NH-$. The general formula for L_x is represented by R_2 . In general R_2 can be $CO-NH$ (amide), $CH(OH)(CH_2)$ (hydroxyethylene), $CH(OH)CH(OH)$ (dihydroxyethylene), $CH(OH)CH_2NH$ (hydroxyethylamine), $PO(OH)CH_2$ (phosphinate), CH_2NH , (reduced amide). It is understood that more than one L - maybe an isostere as long as the substrate analog functions to inhibit aspartic protease function.

L_s which are not isosteres may either be an amide bond or mimetic of an amide bond that is non-hydrolyzable.

5 X and Y represent molecules which are not typically involved in the
recognition by the aspartic protease recognition site, but which do not
interfere with recognition. It is preferred that these molecules confer
10 resistance to the degradation of the substrate analog. Preferred examples
would be amino acids coupled to the substrate analog through a non-
hydrolyzable bond. Other preferred compounds would be capping agents.
Still other preferred compounds would be compounds which could be used
15 in the purification of the substrate analogs such as biotin.

As used herein, alkyl refers to substituted or unsubstituted straight,
20 branched or cyclic alkyl groups; and alkoxy refers to substituted or
unsubstituted straight, branched or cyclic alkoxy. Preferably, alkyl and
alkoxy groups have from 1 to 40 carbons, and more preferably from 1 to 20
carbons, from 1 to 10 carbons, or from 1 to 3 carbons.

As used herein, alkenyl refers to substituted or unsubstituted
25 straight chain or branched alkenyl groups; alkynyl refers to substituted or
unsubstituted straight chain or branched alkynyl groups; alkenyloxy refers to
substituted or unsubstituted straight chain or branched alkenyloxy; and
alkynyloxy refers to substituted or unsubstituted straight chain or branched
30 alkynyloxy. Preferably, alkenyl, alkynyl, alkenyloxy and alkynyloxy groups
have from 2 to 40 carbons, and more preferably from 2 to 20 carbons, from 2
to 10 carbons, or from 2 to 3 carbons.

As used herein, alkaryl refers to an alkyl group that has an aryl
35 substituent; aralkyl refers to an aryl group that has an alkyl substituent;
heterocyclic-alkyl refers to a heterocyclic group with an alkyl substituent;
alkyl-heterocyclic refers to an alkyl group that has a heterocyclic substituent.

40 The substituents for alkyl, alkenyl, alkynyl, alkoxy, alkenyloxy, and
alkynyloxy groups can be halogen, cyano, amino, thio, carboxy, ester, ether,
thioether, carboxamide, hydroxy, or mercapto. Further, the groups can
optionally have one or more methylene groups replaced with a heteroatom,
45 such as O, NH or S.

A number of different substrates were tested and analyzed, and the
50 cleavage rules for Memapsin 2 were determined. The results of the

substrates which were analyzed are presented in Table 1 and the rules determined from these results are summarized below.

(1) The primary specificity site for a memapsin 2 substrate is subsite position, P_1' . This means that the most important determinant for substrate specificity in memapsin 2 is the amino acid, $S1'$. P_1' must contain a small side chain for memapsin 2 to recognize the substrate. Preferred embodiments are substrate analogs where R_1 of the P_1' position is either H (side chain of glycine), CH_3 (side chain of alanine), CH_2OH (side chain of serine), or CH_2OOH (side chain of aspartic acid). Embodiments that have an R_1 structurally smaller than CH_3 (side chain of alanine) or CH_2OH (side chain of serine) are also preferred.

(2) There are no specific sequence requirements at positions P_4 , P_3 , P_2 , P_1 , P_2' , P_3' , and P_4' . Each site can accommodate any other amino acid residue in singularity as long as rule number 3 is met.

(3) At least two of the remaining seven positions, P_4 , P_3 , P_2 , P_1 , P_2' , P_3' , and P_4' , must have an R_1 which is made up of a hydrophobic residue. It is preferred that there are at least three hydrophobic residues in the remaining seven positions, P_4 , P_3 , P_2 , P_1 , P_2' , P_3' , and P_4' . Preferred R_1 groups for the positions that contain a hydrophobic group are CH_3 (side chain of alanine), $CH(CH_3)_2$ (side chain of valine), $CH_2CH(CH_3)_2$ (side chain of leucine), $(CH_3)CH(CH_2CH_3)$ (side chain of isoleucine), $CH_2(INDOLE)$ (side chain of tryptophan), $CH_2(Benzene)$ (side chain of phenylalanine), $CH_2CH_2SCH_3$ (side chain of methionine), $CH_2(Phenol)$ (side chain of tyrosine). It is more preferred that the hydrophobic group be a large hydrophobic group. Preferred R_1 s which contain large hydrophobic groups are $CH(CH_3)_2$ (side chain of valine), $CH_2CH(CH_3)_2$ (side chain of leucine), $(CH_3)CH(CH_2CH_3)$ (side chain of isoleucine), $CH_2(Indole)$ (side chain of tryptophan), $CH_2(Benzene)$ (side chain of phenylalanine), $CH_2CH_2SCH_3$ (side chain of methionine), $CH_2(Phenol)$ (side chain of tyrosine). It is most preferred that positions with a hydrophobic R_1 are $CH(CH_3)_2$ (side chain of valine), $CH_2CH(CH_3)_2$ (side chain of leucine), $CH_2(Benzene)$ (side chain of

5 phenylalanine), $\text{CH}_2\text{CH}_2\text{SCH}_3$ (side chain of methionine), or CH_2 (Phenol)
(side chain of tyrosine).

(4) None of the eight positions, P_4 , P_3 , P_2 , P_1 , P_1' , P_2' , P_3' , and P_4' may have
a proline side chain at its R1 position.

10 (5) Not all subsites must have an P represented in the analog. For example,
a substrate analog could have $\text{X-P}_2\text{-L}_1\text{-P}_1\text{-L}_0\text{-P}_1'\text{-L}_1'\text{-P}_2'\text{-L}_2'\text{-P}_3'\text{-L}_3'\text{-Y}$ or it
could have $\text{X-L}_1\text{-P}_1\text{-L}_0\text{-P}_1'\text{-L}_1'\text{-P}_2'\text{-L}_2'\text{-P}_3'\text{-L}_3'\text{-P}_4'\text{-L}_4'\text{-Y}$.

15 Preferred substrate analogs are analogs having the sequences
disclosed in Table 1, with the non-hydrolyzable analog between P_1 and P_1' .

Combinatorial Chemistry to Make Inhibitors

20 Combinatorial chemistry includes but is not limited to all methods for
isolating molecules that are capable of binding either a small molecule or
another macromolecule. Proteins, oligonucleotides, and polysaccharides are
examples of macromolecules. For example, oligonucleotide molecules with
25 a given function, catalytic or ligand-binding, can be isolated from a complex
mixture of random oligonucleotides in what has been referred to as "*in vitro*
genetics" (Szostak, TIBS 19:89, 1992). One synthesizes a large pool of
molecules bearing random and defined sequences and subjects that complex
30 mixture, for example, approximately 10^{15} individual sequences in 100 μg of
a 100 nucleotide RNA, to some selection and enrichment process. Through
repeated cycles of affinity chromatography and PCR amplification of the
molecules bound to the ligand on the column, Ellington and Szostak (1990)
35 estimated that 1 in 10^{10} RNA molecules folded in such a way as to bind a
small molecule dyes. DNA molecules with such ligand-binding behavior
have been isolated as well (Ellington and Szostak, 1992; Bock et al, 1992).

40 Techniques aimed at similar goals exist for small organic molecules,
proteins and peptides and other molecules known to those of skill in the art.
Screening sets of molecules for a desired activity whether based on libraries
of small synthetic molecules, oligonucleotides, proteins or peptides is
45 broadly referred to as combinatorial chemistry.

There are a number of methods for isolating proteins either have *de*
novo activity or a modified activity. For example, phage display libraries

5 have been used for a number of years. A preferred method for isolating
proteins that have a given function is described by Roberts and Szostak
(Roberts R.W. and Szostak J.W. Proc. Natl. Acad. Sci. USA, 94(23)12997-
10 302 (1997). Another preferred method for combinatorial methods designed
to isolate peptides is described in Cohen et al. (Cohen B.A., et al., Proc. Natl.
Acad. Sci. USA 95(24):14272-7 (1998)). This method utilizes a modified
15 two-hybrid technology. Yeast two-hybrid systems are useful for the
detection and analysis of protein:protein interactions. The two-hybrid
system, initially described in the yeast *Saccharomyces cerevisiae*, is a
powerful molecular genetic technique for identifying new regulatory
20 molecules, specific to the protein of interest (Fields and Song, *Nature*
340:245-6 (1989)). Cohen et al., modified this technology so that novel
interactions between synthetic or engineered peptide sequences could be
identified which bind a molecule of choice. The benefit of this type of
25 technology is that the selection is done in an intracellular environment. The
method utilizes a library of peptide molecules that attach to an acidic
activation domain. A peptide of choice, for example an extracellular portion
of memapsin 2 is attached to a DNA binding domain of a transcriptional
30 activation protein, such as Gal 4. By performing the Two-hybrid technique
on this type of system, molecules that bind the extracellular portion of
memapsin 2 can be identified.

Screening of Small Molecule Libraries

35 In addition to these more specialized techniques, methodology well
known to those of skill in the art, in combination with various small
molecule or combinatorial libraries, can be used to isolate and characterize
40 those molecules which bind to or interact with the desired target, either
memapsin 2 or its substrate. The relative binding affinity of these
compounds can be compared and optimum inhibitors identified using
competitive or non-competitive binding studies which are well known to
45 those of skill in the art. Preferred competitive inhibitors are non-
hydrolyzable analogs of memapsin 2. Another will cause allosteric

5 rearrangements which prevent memapsin 2 from functioning or folding correctly.

Computer assisted Rational Drug Design

10 Another way to isolate inhibitors is through rational design. This is achieved through structural information and computer modeling. Computer modeling technology allows visualization of the three-dimensional atomic structure of a selected molecule and the rational design of new compounds that will interact with the molecule. The three-dimensional construct
15 typically depends on data from x-ray crystallographic analyses or NMR imaging of the selected molecule. The molecular dynamics require force field data. The computer graphics systems enable prediction of how a new compound will link to the target molecule and allow experimental manipulation of the structures of the compound and target molecule to perfect binding specificity. For example, using NMR spectroscopy, Inouye and coworkers were able to obtain the structural information of N-terminal
20 truncated TSHK (transmembrane sensor histidine kinases) fragments which retain the structure of the individual sub-domains of the catalytic site of a TSHK. On the basis of the NMR study, they were able to identify potential TSHK inhibitors (U.S. Patent No. 6,077,682 to Inouye). Another good example is based on the three-dimensional structure of a calcineurin/FKBP12/FK506 complex determined using high resolution X-ray crystallography to obtain the shape and structure of both the calcineurin
35 active site binding pocket and the auxiliary FKBP12/FK506 binding pocket (U.S. Patent No. 5,978,740 to Armistead). With this information in hand, researchers can have a good understanding of the association of natural ligands or substrates with the binding pockets of their corresponding
40 receptors or enzymes and are thus able to design and make effective inhibitors.

45 Prediction of molecule-compound interaction when small changes are made in one or both requires molecular mechanics software and computationally intensive computers, usually coupled with user-friendly, menu-driven interfaces between the molecular design program and the user.

5 Examples of molecular modeling systems are the CHARMM and QUANTA
programs. Polygen Corporation, Waltham, MA. CHARMM performs the
energy minimization and molecular dynamics functions. QUANTA
10 performs the construction, graphic modeling and analysis of molecular
structure. QUANTA allows interactive construction, modification,
visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive
15 with specific proteins, such as Rotivinen, et al., 1988 *Acta Pharmaceutica
Fennica* 97, 159-166; Ripka, *New Scientist* 54-57 (June 16, 1988); McKinaly
and Rossmann, 1989 *Annu. Rev. Pharmacol. Toxicol.* 29, 111-122; Perry
and Davies, QSAR: Quantitative Structure-Activity Relationships in Drug
20 Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 1989 *Proc.
R. Soc. Lond.* 236, 125-140 and 141-162; and, with respect to a model
enzyme for nucleic acid components, Askew, et al., 1989 *J. Am. Chem. Soc.*
111, 1082-1090. Other computer programs that screen and graphically
25 depict chemicals are available from companies such as BioDesign, Inc.,
Pasadena, CA., Allelix, Inc, Mississauga, Ontario, Canada, and Hypercube,
Inc., Cambridge, Ontario.

30 Although described above with reference to design and generation of
compounds which could alter binding, one could also screen libraries of
known compounds, including natural products or synthetic chemicals, and
biologically active materials, including proteins, for compounds which alter
35 substrate binding or enzymatic activity.

Screening of Libraries

Design of substrate analogs and rational drug design are based on
40 knowledge of the active site and target, and utilize computer software
programs that create detailed structures of the enzyme and its substrate, as
well as ways they interact, alone or in the presence of inhibitor. These
techniques are significantly enhanced with x-ray crystallographic data in
45 hand. Inhibitors can also be obtained by screening libraries of existing
compounds for those which inhibit the catalytically active enzyme. In
contrast to reports in the literature relating to memapsin 2, the enzyme

described herein has activity analogous to the naturally produced enzyme, providing a means for identifying compounds which inhibit the endogenous activity. These potential inhibitors are typically identified using high throughput assays, in which enzyme, substrate (preferably a chromogenic substrate) and potential inhibitor (usually screened across a range of concentrations) are mixed and the extent of cleavage of substrate determined. Potentially useful inhibitors are those which decrease the amount of cleavage.

III. Methods of diagnosis and treatment

Inhibitors can be used in the diagnosis and treatment and/or prevention of Alzheimer's disease and conditions associated therewith, such as elevated levels of the forty-two amino acid peptide cleavage product, and the accumulation of the peptide in amyloid plaques.

Diagnostic Uses

The substrate analogs can be used as reagents for specifically binding to memapsin 2 or memapsin 2 analogs and for aiding in memapsin 2 isolation and purification or characterization, as described in the examples. The inhibitors and purified recombinant enzyme can be used in screens for those individuals more genetically prone to develop Alzheimer's disease.

Therapeutic Uses

Recombinant human memapsin 2 cleaves a substrate with the sequence LVNM/AEGD (SEQ ID NO:9). This sequence is the *in vivo* processing site sequence of human presenilins. Both presenilin 1 and presenilin 2 are integral membrane proteins. They are processed by protease cleavage, which removes the N terminal sequence from the unprocessed form. Once processed, presenilin forms a two-chain heterodimer (Capell et al., J. Biol. Chem. 273, 3205 (1998); Thinakaran et al., Neurobiol. Dis. 4, 438 (1998); Yu et al., Neurosci Lett. 2:254(3):125-8 (1998)), which is stable relative to the unprocessed presenilins. Unprocessed presenilins are quickly degraded (Thinakaran et al., J. Biol. Chem. 272, 28415 (1997); Steiner et al., J. Biol. Chem. 273, 32322 (1998)). It is known that presenilin controls the *in vivo* activity of beta-secretase, which in turn cleaves the amyloid precursor

5 protein (APP) leading to the formation of alpha-beta42. The accumulation of
alpha-beta42 in the brain cells is known to be a major cause of Alzheimer's
10 disease (for review, see Selkoe, 1998). The activity of presenilin therefore
enhances the progression of Alzheimer's disease. This is supported by the
observation that in the absence of presenilin gene, the production of alpha-
beta42 peptide is lowered (De Strooper et al., Nature 391, 387 (1998)).
15 Since unprocessed presenilin is degraded quickly, the processed,
heterodimeric presenilin must be responsible for the accumulation of alpha-
beta42 leading to Alzheimer's disease. The processing of presenilin by
memapsin 2 would enhance the production of alpha-beta42 and therefore,
20 further the progress of Alzheimer's disease. Therefore a memapsin 2
inhibitor that crosses the blood brain barrier can be used to decrease the
likelihood of developing or slow the progression of Alzheimer's disease
which is mediated by deposition of alpha-beta42. Since memapsin 2 cleaves
25 APP at the beta cleavage site, prevention of APP cleavage at the beta
cleavage site will prevent the build up of alpha-beta42.

Vaccines

30 The catalytically active memapsin 2 or fragments thereof including
the active site defined by the presence of two catalytic aspartic residues and
substrate binding cleft can be used to induce an immune response to the
memapsin 2. The memapsin 2 is administered in an amount effective to
35 elicit blocking antibodies, i.e., antibodies which prevent cleavage of the
naturally occurring substrate of memapsin 2 in the brain. An unmodified
vaccine may be useful in the prevention and treatment of Alzheimer's
disease. The response to the vaccine may be influenced by its composition,
40 such as inclusion of an adjuvant, viral proteins from production of the
recombinant enzyme, and/or mode of administration (amount, site of
administration, frequency of administration, etc). Since it is clear that the
enzyme must be properly folded in order to be active, antibody should be
45 elicited that is active against the endogenous memapsin 2. Antibodies that
are effective against the endogenous enzyme are less likely to be produced
against the enzyme that is not properly refolded.

Pharmaceutically Acceptable Carriers

The inhibitors will typically be administered orally or by injection. Oral administration is preferred. Alternatively, other formulations can be used for delivery by pulmonary, mucosal or transdermal routes. The inhibitor will usually be administered in combination with a pharmaceutically acceptable carrier. Pharmaceutical carriers are known to those skilled in the art. The appropriate carrier will typically be selected based on the mode of administration. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, and analgesics.

Preparations for parenteral administration or administration by injection include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Preferred parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, and electrolyte replenishers (such as those based on Ringer's dextrose).

Formulations for topical (including application to a mucosal surface, including the mouth, pulmonary, nasal, vaginal or rectal) administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Formulations for these applications are known. For example, a number of pulmonary formulations have been developed, typically using spray drying to formulate a powder having particles with an aerodynamic diameter of between one and three microns, consisting of drug or drug in combination with polymer and/or surfactant.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable.

5 Peptides as described herein can also be administered as a
pharmaceutically acceptable acid- or base- addition salt, formed by reaction
with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric
10 acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and
organic acids such as formic acid, acetic acid, propionic acid, glycolic acid,
lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic
15 acid, and fumaric acid, or by reaction with an inorganic base such as sodium
hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases
such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

Dosages

20 Dosing is dependent on severity and responsiveness of the condition to
be treated, but will normally be one or more doses per day, with course of
treatment lasting from several days to several months or until the attending
physician determines no further benefit will be obtained. Persons of ordinary
25 skill can determine optimum dosages, dosing methodologies and repetition
rates.

30 The dosage ranges are those large enough to produce the desired
effect in which the symptoms of the memapsin 2 mediated disorder are
alleviated (typically characterized by a decrease in size and/or number of
amyloid plaque, or by a failure to increase in size or quantity), or in which
cleavage of the alpha-beta42 peptide is decreased. The dosage can be
35 adjusted by the individual physician in the event of any counterindications.

The present invention will be further understood by reference to the
following non-limiting examples.

Example 1. Cloning of memapsin 2.

1. Cloning and nucleotide sequence of pro-memapsin 2.

40 New sequences homologous to human aspartic proteases were found
in the following entries in the EST IMAGE database: AA136368 pregnant
uterus ATCC 947471, AA207232 neurepithelium ATCC 214526, and
45 R55398 human breast ATCC 392689. The corresponding bacterial strains:
#947471, #214526, and # 392689 containing the EST sequences were
obtained from the ATCC (Rockville, MD). The sequencing of these clones

obtained from ATCC confirmed that they contained sequences not identical to known human aspartic proteases. The completed sequences of these clones assembled into about 80% of prepro-M2 cDNA. Full length cDNAs of these clones were obtained using the following methods.

The Human Pancreas Marathon-Ready cDNA (Clontech), which is double-strand cDNA obtained by reverse-transcription, primer addition, and second strand synthesis of mRNA from human tissues, was used as template for PCR amplification. An adapter primer (AP1) and a nested adapter primer (AP2) were used for 5'- and 3'-RACE PCR. For PCR the 5'-region of the memapsin 2 cDNA, primers AP1 and NHASPR1 were used. Primers for the 3'-end of the cDNA are NHASPF2 and AP1. The middle of the cDNA was amplified by primers NHASPF1 and NHASPR2. The sequence for the primers is as follows: NHASPF1:

GGTAAGCATCCCCCATGGCCCCAACGTC (SEQ ID NO:10),

NHASPR1: GACGTTGGGGCCATGGGGGATGCTTACC (SEQ ID NO:11),

NHASPF2: ACGTTGTCTTTGATCGGGCCCGAAAACGAATTGG (SEQ ID NO:12),

NHASPR2: CCAATTCGTTTTTCGGGCCCCGATCAAAGACAACG (SEQ ID NO:13),

AP1: CCATCCTAATACGACTCACTATAGGGC (SEQ ID NO:14), and

AP2: ACTCACTATAGGGCTCGAGCGGC (SEQ ID NO:15)

Memapsin 2 was also cloned from a human pancreas library (Quick-Screen Human cDNA Library Panel) contained in lambda-gt10 and lambda-gt11 vectors. The primers from the vectors, GT10FWD, GT10REV, GT11FWD, and GT11REV, were used as outside primers. The sequence of the primers used was:

GT10FWD: CTTTGTAGCAAGTTCAGCCTGGTTAA (SEQ ID NO:16),

GT10REV: GAGGTGGCTTATGAGTATTTCTCCAGGGTA (SEQ ID NO:17),

GT11FWD: TGGCGACGACTCCTGGAGCCCCG (SEQ ID NO:18),

GT11REV: TGACACCAGACCAACTGGTAATGG (SEQ ID NO:19).

5 In addition, memapsin 2 cDNA was amplified directly from the human pancreatic lambda-gt10 and lambda-gt11 libraries. The sequence of the primers was: PASPN1: catatgGCGGGAGTGCTGCCTGCCAC (SEQ ID NO:20) and
10 NHASPC1: ggatccTCACTTCAGCAGGGAGATGTCATCAGCAAAGT (SEQ ID NO:21).

15 The amplified memapsin 2 fragments were cloned into an intermediate PCR vector (Invitrogen) and sequenced.

The assembled cDNA from the fragments, the nucleotide and the deduced protein sequence are shown in SEQ ID NO 1 and SEQ ID NO 2.

20 Pro-memapsin 2 is homologous to other human aspartic proteases. Based on the alignments, Pro-memapsin 2 contains a *pro* region, an aspartic protease region, and a trans-membrane region near the C-terminus. The active enzyme is memapsin 2 and its pro-enzyme is pro-memapsin 2.

25 **Example 2. Distribution of memapsin 2 in human tissues.**

Multiple tissue cDNA panels from Clontech were used as templates for PCR amplification of a 0.82 kb fragment of memapsin 2 cDNA. The primers used for memapsin 2 were NHASPF1 and NHASPR2. Tissues that
30 contain memapsin 2 or fragments of memapsin 2 yielded amplified PCR products. The amount of amplified product indicated that memapsin 2 is present in the following organs from most abundant to least abundant: pancreas, brain, lung, kidney, liver, placenta, and heart. Memapsin 2 is also
35 present in spleen, prostate, testis, ovary, small intestine, and colon cells.

Example 3. Expression of pro-memapsin 2 cDNA in E. coli, refolding and purification of pro-memapsin 2.

40 The pro-memapsin 2 was PCR amplified and cloned into the *Bam*HI site of a pET11a vector. The resulting vector expresses pro-memapsin 2 having a sequence from Ala-8p to Ala 326. Figure 1 shows the construction of two expression vectors, pET11-memapsin 2-T1 (hereafter T1) and pET11-memapsin 2-T2 (hereafter T2). In both vectors, the N-terminal 15 residues
45 of the expressed recombinant proteins are derived from the expression vector. Pro-memapsin 2 residues start at residue Ala-16. The two

5 recombinant pro-memapsin 2s have different C-terminal lengths. Clone T1 ends at Thr- 454 and clone T2 ends at Ala-419. The T1 construct contains a C-terminal extension from the T2 construct but does not express any of the predicted transmembrane domain.

10 *Expression of recombinant proteins and recovery of inclusion bodies*

The T1 and T2 expression vectors were separately transfected into *E. coli* strain BL21(DE3). The procedures for the culture of transfected bacteria, induction for synthesis of recombinant proteins and the recovery and washing of inclusion bodies containing recombinant proteins are essentially as previously described (Lin et al., 1994).

Three different refolding methods have produced satisfactory results.

20 *(i) The rapid dilution method.*

Pro-memapsin 2 in 8 M urea/100 mM beta-mercaptoethanol with $OD_{280nm} = 5$ was rapidly diluted into 20 volumes of 20 mM-Tris, pH 9.0. The solution was slowly adjusted into pH 8 with 1 M HCl. The refolding solution was then kept at 4° C for 24 to 48 hours before proceeding with purification.

30 *(ii) The reverse dialysis method*

An equal volume of 20 mM Tris, 0.5 mM oxidized/1.25 mM reduced glutathione, pH 9.0 is added to rapidly stirred pro-memapsin 2 in 8 M urea/10 mM beta-mercaptoethanol with $OD_{280nm} = 5$. The process is repeated three more times with 1 hour intervals. The resulting solution is then dialyzed against sufficient volume of 20 mM Tris base so that the final urea concentration is 0.4 M. The pH of the solution is then slowly adjusted to 8.0 with 1 M HCl.

40 *iii. The preferred method for refolding.*

Inclusion bodies are dissolved in 8 M urea, 0.1 M Tris, 1 mM Glycine, 1 mM EDTA, 100 mM beta-mercaptoethanol, pH 10.0. The OD_{280} of the inclusion bodies are adjusted to 5.0 with the 8 M urea solution without beta-mercaptoethanol. The final solution contains the following reducing reagents:

5 10 mM beta-mercaptoethanol, 10 mM DTT (Dithiothreitol), 1 mM reduced glutathion, and 0.1 M oxidized glutathion. The final pH of the solution is 10.0.

10 The above solution is rapidly diluted into 20 volumes of 20 mM Tris base, the pH is adjusted to 9.0, and the resulting solution is kept at 4 °C for 16 hr. The solution is equilibrated to room temperature in 6 hr. and the pH is adjusted to 8.5. The solution is returned to 4 °C again for 18 hr.

15 The solution is again equilibrated to room temperature in 6 hr. and the pH is adjusted to 8.0. The solution is returned to 4 °C again for 4 to 7 days.

20 The refolding procedures are critical to obtain an enzymically active preparation which can be used for studies of subsite specificity of M2, to analyze inhibition potency of M2 inhibitors, to screen for inhibitors using either random structural libraries or existing collections of compound libraries, to produce crystals for crystallography studies of M2 structures, 25 and to produce monoclonal or polyclonal antibodies of M2.

Purification of recombinant pro-memapsin 2-T2

30 The refolded material is concentrated by ultrafiltration, and separated on a SEPHACRYL™ S-300 column equilibrated with 20 mM Tris.HCl, 0.4 M urea, pH 8.0. The refolded peak (second peak) from the S-300 column can be further purified with a FPLC RESOURCE-Q™ column, which is equilibrated with 20 mM Tris-HCl, 0.4 M urea, pH 8.0. The enzyme is eluted 35 from the column with a linear gradient of NaCl. The refolded peak from S-300 can also be activated before further purification. For activation, the fractions are mixed with equal volume 0.2 M Sodium Acetate, 70% glycerol, pH 4.0. The mixture is incubated at 22 °C for 18 hr. and then dialyzed twice 40 against 20 volumes of 20 mM Bis-Tris, 0.4 M urea, pH 6.0. The dialyzed materials are then further purified on a FPLC RESOURCE-Q™ column equilibrated with 20 Bis-Tris, 0.4 M urea, pH 6.0. The enzyme is eluted with 45 a linear gradient of NaCl.

50 SDS-PAGE analysis of the S-300 fractions under reduced and non-reduced conditions indicated that Pro-memapsin 2 first elutes as a very high

5 molecular weight band (greater than about 42 kD) under non-reduced
conditions. This indicates that the protein is not folded properly in these
fractions, due to disulfide cross linking of proteins. Subsequent fractions
10 contain a protein of predicted pro-memapsin 2-T2 size (about 42 kDa). The
pro-enzyme obtained in these fractions is also proteolytically active for auto-
catalyzed activation. These fractions were pooled and subjected to
chromatography on the FPLC RESOURCETM column eluted with a linear
15 gradient of NaCl. Some fractions were analyzed using SDS-PAGE under
non-reducing conditions. The analysis showed that fractions 6 and 7
contained most of the active proteins, which was consistent with the first
FPLC peak containing the active protein. The main peak was coupled to a
20 shoulder peak, and was present with repeated purification with the same
RESOURCETM Q column. The main shoulder peaks were identified as
active pro-memapsin 2 that exist in different conformations under these
conditions.

25 **Example 4. Proteolytic activity and cleavage-site preferences of
recombinant memapsin 2.**

The amino acid sequence around the proteolytic cleavage sites was
30 determined in order to establish the specificity of memapsin 2. Recombinant
pro-memapsin 2-T1 was incubated in 0.1 M sodium acetate, pH 4.0, for 16
hours at room temperature in order to create autocatalyzed cleavages. The
products were analyzed using SDS-polyacrylamide gel electrophoresis.
35 Several bands which corresponded to molecular weights smaller than that of
pro-memapsin 2 were observed. The electrophoretic bands were trans-blotted
onto a PVDF membrane. Four bands were chosen and subjected to N-
terminal sequence determination in a Protein Sequencer. The N-terminal
40 sequence of these bands established the positions of proteolytic cleavage
sites on pro-memapsin 2.

In addition, the oxidized B-chain of bovine insulin and two different
45 synthetic peptides were used as substrates for memapsin 2 to determine the
extent of other hydrolysis sites. These reactions were carried out by auto-
activated pro-memapsin 2 in 0.1 M sodium acetate, pH 4.0, which was then

incubated with the peptides. The hydrolytic products were subjected to HPLC on a reversed phase C-18 column and the eluent peaks were subjected to electrospray mass spectrometry for the determination of the molecular weight of the fragments. Two hydrolytic sites were identified on oxidized insulin B-chain (Table 1). Three hydrolytic sites were identified from peptide NCII-gamma. A single cleavage site was observed in synthetic peptide PSI-gamma, whose sequence (LVNMAEGD) (SEQ ID NO:9) is derived from the beta-processing site of human presenilin 1 (Table 1).

Table 1: Substrate Specificity of Memapsin 2

Site #	Substrate	P4	P3	P2	P1	P1'	P2'	P3'	P4'	
1	Pro-memapsin 2	R	G	S	M	A	G	V	L	aa 12-18 of SEQ ID No.3
2		G	T	Q	H	G	I	R	L	aa 23-30 of SEQ ID No. 3
3		S	S	N	F	A	V	G	A	aa 98-105 of SEQ ID No. 3
4		G	L	A	Y	A	E	I	A	aa 183-190 of SEQ ID No.3
5	Oxidized insulin B-chain *	H	L	C [^]	G	S	H	L	V	C [^] is cysteic acid: SEQ ID No. 22 SEQ ID No. 23
6		C [^]	G	E	R	G	F	F	Y	
7	Synthetic peptide*				V	G	S	G	V	Three sites cleaved in a peptide VGSGVLLSRK (SEQ ID NO:30) SEQ ID No. 24 SEQ ID No. 25 SEQ ID No. 26
8			V	G	S	G	V	L	L	
9		G	V	L	L	S	R	K		
10	Peptide**	L	V	N	M	A	E	G	D	SEQ ID No. 9

Example 5. Activation of pro-memapsin 2 and enzyme kinetics.

Incubation in 0.1 M sodium acetate, pH 4.0, for 16 h at 22°C autocatalytically converted *pro*-M2_{pd} to M2_{pd}. For initial hydrolysis tests, two synthetic peptides were separately incubated with *pro*-M2_{pd} in 0.1 M Na acetate, pH 4.0 for different periods ranging from 2 to 18 h. The incubated samples were subjected to LC/MS for the identification of the hydrolytic products. For kinetic studies, the identified HPLC (Beckman System Gold) product peaks were integrated for quantitation. The K_m and k_{cat} values for presenilin 1 and Swedish APP peptides (Table 1) were measured by steady-state kinetics. The individual K_m and k_{cat} values for APP peptide could not be measured accurately by standard methods, so its k_{cat}/K_m value was measured by competitive hydrolysis of mixed substrates against presenilin 1 peptide (Fersht, A. "Enzyme Structure and Mechanism", 2nd Ed., W.H. Freeman and Company, New York, (1985)).

The results are shown in Figures 2A and 2B. The conversion of *pro*-M2_{pd} at pH 4.0 to smaller fragments was shown by SDS-polyacrylamide electrophoresis. The difference in migration between *pro*-M2_{pd} and converted enzyme is evident in a mixture of the two. Figure 2A is a graph of the initial rate of hydrolysis of synthetic peptide swAPP (see Table 1) by M2_{pd} at different pH. Figure 2B is a graph of the relative k_{cat}/K_m values for steady-state kinetic of hydrolysis of peptide substrates by M2_{pd}.

Example 6. Expression in Mammalian cells.Methods

PM2 cDNA was cloned into the *EcoRV* site of vector pSecTag A (Invitrogen). Human APP cDNA was PCR amplified from human placenta 8-g111 library (Clontech) and cloned into the *NheI* and *XbaI* sites of pSecTag A. The procedure for transfection into HeLa cells and vaccinia virus infection for T7-based expression are essentially the same as described by Lin, X., *FASEB J.* 7:1070-1080 (1993).

Transfected cells were metabolically labeled with 200 microCi ³⁵S methionine and cysteine (TransLabel; ICN) in 0.5 ml of serum-free/methionine-free media for 30 min, rinsed with 1 ml media, and replaced

5 with 2 ml DMEM/10% FCS. In order to block vesicle acidification.
Bafilomycin A1 was included in the media (Perez, R.G., et al., *J Biol. Chem*
271:9100-9107 (1996)). At different time points (chase), media was
10 removed and the cells were harvested and lysed in 50 mM Tris, 0.3 M NaCl,
5 mM EDTA, 1% Triton X-100, pH 7.4, containing 10 mM iodoacetamide,
10 :M TPCK, 10 :M TLCK, and 2 microg/ml leupeptin. The supernatant
(14,000 x g) of cell lysates and media were immunoabsorbed onto antibody
15 bound to protein G sepharose (Sigma). Anti-APP N-terminal domain
antibody (Chemicon) was used to recover the betaN-fragment of APP and
anti-alpha-beta₁₋₁₇ antibody (Chemicon, recognizing the N-terminal 17
residues of alpha-beta) was used to recover the 12 kDa β C-fragment. The
20 former antibody recognized only denatured protein, so media was first
incubated in 2 mM dithiothreitol 0.1% SDS at 55°C for 30 min before
immunoabsorption. Samples were cooled and diluted with an equal volume
of cell lysis buffer before addition of anti-APP N-terminal domain
25 (Chemicon). Beads were washed, eluted with loading buffer, subjected to
SDS-PAGE (NOVEXTM) and visualized by autoradiogram or
phosphorimaging (Molecular Dynamics) on gels enhanced with Amplify
(Amersham). Immunodetection of the betaN-fragment was accomplished by
30 transblotting onto a PVDF membrane and detecting with anti-alpha-beta₁₋₁₇
and chemiluminescent substrate (Amersham).

35 Results.

HeLa cells transfected with APP or M2 in 4-well chamber slides were
fixed with acetone for 10 min and permeabilized in 0.2% Triton X-100 in
PBS for 6 min. For localizing M2, polyclonal goat anti-*pro*-M2_{pd} antibodies
40 were purified on DEAE-sepharose 6B and affinity purified against
recombinant *pro*-M2_{pd} immobilized on Affigel (BioRad). Purified anti-*pro*-
M2_{pd} antibodies were conjugated to Alexa568 (Molecular Probes) according
to the manufacturer's protocol. Fixed cells were incubated overnight with a
45 1:100 dilution of antibody in PBS containing 0.1% BSA and washed 4 times
with PBS. For APP, two antibodies were used. Antibody A β ₁₋₁₇ (described
above) and antibody A β ₁₇₋₄₂, which recognizes the first 26 residues

5 following the beta-secretase cleavage site (Chemicon). After 4 PBS washes,
the cells were incubated overnight with an anti-mouse FITC conjugate at a
dilution of 1:200. Cells were mounted in Prolong anti-fade reagent
10 (Molecular Probes) and visualized on a Leica TCS confocal laser scanning
microscope.

Example 7: Design and Synthesis of OM99-1 and OM99-2.

Based on the results of specificity studies of memapsin 2, it was
15 predicted that good residues for positions P1 and P1' would be Leu and Ala.
It was subsequently determined from the specificity data that P1' preferred
small residues, such as Ala and Ser. However, the crystal structure
(determined below in Example 9) indicates that this site can accommodate a
20 lot of larger residues. It was demonstrated that P1' of memapsin 2 is the
position with the most stringent specificity requirement where residues of
small side chains, such as Ala, Ser, and Asp, are preferred. Ala was selected
for P1' mainly because its hydrophobicity over Ser and Asp is favored for the
25 penetration of the blood-brain barrier, a requirement for the design of a
memapsin 2 inhibitor drug for treating Alzheimer's disease. Therefore,
inhibitors were designed to place a transition-state analogue isostere between
Leu and Ala (shown as Leu*Ala, where * represents the transition-state
30 isostere, -CH(OH)-CH₂-) and the subsite P4, P3, P2, P2', P3' and P4' are
filled with the beta-secretase site sequence of the Swedish mutant from the
beta-amyloid protein. The structures of inhibitors OM99-1 and OM99-2 are
35 shown below and in Figures 3A and 3B, respectively:

OM99-1: Val-Asn-Leu*Ala-Ala-Glu-Phe (SEQ. ID NO. 27)
40 OM99-2: Glu-Val-Asn-Leu*Ala-Ala-Glu-Phe (SEQ. ID NO. 28)

The Leu*Ala dipeptide isostere was synthesized as follows:

45 The Leu-Ala dipeptide isostere for the M₂-inhibitor was prepared
from L-leucine. As shown in Scheme 1, L-leucine was protected as its BOC-
derivative 2 by treatment with BOC₂O in the presence of 10% NaOH in
diethyl ether for 12 h. Boc-leucine 2 was then converted to Weinreb amide 3

5 by treatment with isobutyl chloroformate and N-methylpiperidine followed
by treatment of the resulting mixed anhydride with N,O-
dimethylhydroxylamine (Nahm and Weinreb, Tetrahedron Letters 1981, 32,
3815). Reduction of 3 with lithium aluminum hydride in diethyl ether
10- provided the aldehyde 4. Reaction of the aldehyde 4 with lithium propiolate
derived from the treatment of ethyl propiolate and lithium diisopropylamide
afforded the acetylenic alcohol 5 as an inseparable mixture of diastereomers
15 (5.8:1) in 42% isolated yield (Fray, Kaye and Kleinman, J. Org. Chem. 1986,
51, 4828-33). Catalytic hydrogenation of 5 over Pd/BaSO₄ followed by
acid-catalyzed lactonization of the resulting gamma-hydroxy ester with a
catalytic amount of acetic acid in toluene at reflux, furnished the gamma-
20 lactone 6 and 7 in 73% yield. The isomers were separated by silica gel
chromatography by using 40% ethyl acetate in hexane as the eluent.
Introduction of the methyl group at C-2 was accomplished by stereoselective
alkylation of 7 with methyl iodide (Scheme 2). Thus, generation of the
25 dianion of lactone 7 with lithium hexamethyldisilazide (2.2 equivalents) in
tetrahydrofuran at -78°C (30 min) and alkylation with methyl iodide (1.1
equivalents) for 30 min at -78°C, followed by quenching with propionic
acid (5 equivalents), provided the desired alkylated lactone 8 (76% yield)
30 along with a small amount (less than 5%) of the corresponding epimer
(Ghosh and Fidanze, 1998 J. Org. Chem. 1998, 63, 6146-54). The epimeric
cis-lactone was removed by column chromatography over silica gel using a
mixture (3:1) of ethyl acetate and hexane as the solvent system. The
35 stereochemical assignment of alkylated lactone 8 was made based on
extensive ¹H-NMR NOE experiments. Aqueous lithium hydroxide
promoted hydrolysis of the lactone 8 followed by protection of the gamma-
hydroxyl group with *tert*-butyldimethylsilyl chloride in the presence of
imidazole and dimethylaminopyridine in dimethylformamide afforded the
40 acid 9 in 90% yield after standard work-up and chromatography. Selective
removal of the BOC-group was effected by treatment with trifluoroacetic
acid in dichloromethane at 0°C for 1 h. The resulting amine salt was then
reacted with commercial (Aldrich, Milwaukee) Fmoc-succinimide derivative
50

5 in dioxane in the presence of aqueous NaHCO_3 to provide the Fmoc-protected L*A isostere 10 in 65% yield after chromatography. Protected isostere 10 was utilized in the preparation of a random sequence inhibitor library.

10- **Experimental procedure**

N-(*tert*-Butoxycarbonyl)-L-Leucine (2).

To the suspension of 10 g (76.2 mmol) of L-leucine in 140 mL of diethyl ether was added 80 mL of 10 % NaOH. After all solid dissolves, 20 mL (87.1 mmol) of BOC_2O was added to the reaction mixture. The resulting reaction mixture was stirred at 23°C for 12 h. After this period, the layers were separated and the aqueous layer was acidified to pH 1 by careful addition of 1 N aqueous HCl at 0 °C. The resulting mixture was extracted with ethyl acetate (3 x 100 mL). The organic layers were combined and washed with brine and dried over anhydrous Na_2SO_4 . The solvent was removed under reduced pressure to provide title product which was used directly for next reaction without further purification (yield, 97 %). ^1H NMR (400 MHz, CDCl_3) δ 4.89 (broad d, 1H, J = 8.3 Hz), 4.31 (m, 1H), 1.74-1.49 (m, 3H), 1.44 (s, 9H), 0.95 (d, 6H, J = 6.5 Hz).

30 *N*-(*tert*-Butoxycarbonyl)-L-leucine-*N*'-methoxy-*N*'-methylamide (3).

To a stirred solution of N,O-dimethylhydroxyamine hydrochloride (5.52 g, 56.6 mmol) in dry dichloromethane (25 mL) under N_2 atmosphere at 0°C, N-methylpiperidine (6.9 mL, 56.6 mmol) was added dropwise. The resulting mixture was stirred at 0°C for 30 min. In a separate flask, N-(*tert*-butoxycarbonyl)-L-leucine (1) (11.9 g, 51.4 mmol) was dissolved in a mixture of THF (45 mL) and dichloromethane (180 mL) under N_2 atmosphere. The resulting solution was cooled to -20°C. To this solution was added 1-methylpiperidine (6.9 mL, 56.6 mmol) followed by isobutyl chloroformate (7.3 mL, 56.6 mmol). The resulting mixture was stirred for 5 minutes at -20°C and the above solution of N,O-dimethylhydroxyamine was added to it. The reaction mixture was kept -20 °C for 30 minutes and then warmed to 23°C. The reaction was quenched with water and the layers were separated. The aqueous layer was extracted with dichloromethane (3 x 100

5 mL). The combined organic layers were washed with 10% citric acid, saturated sodium bicarbonate, and brine. The organic layer was dried over anhydrous Na₂SO₄ and concentrated under the reduced pressure. The residue was purified by flash silica gel chromatography (25% ethyl acetate/hexane) to yield the title compound 3 (13.8 g, 97%) as a pale yellow oil. ¹H NMR (400 MHz, CDCl₃) δ 5.06 (broad d, 1H, J = 9.1 Hz), 4.70 (m, 1H), 3.82 (s, 3H), 3.13 (s, 3H), 1.70 (m, 1H), 1.46-1.36 (m, 2H), 1.41 (s, 9H), 0.93 (dd, 6H, J = 6.5, 14.2 Hz).

N-(*tert*-Butoxycarbonyl)-*L*-leucinal (4).

To a stirred suspension of lithium aluminum hydride (770 mg, 20.3 mmol) in dry diethyl ether (60 mL) at -40 °C under N₂ atmosphere, was added *N*-*tert*-butoxycarbonyl-*L*-leucine-*N*'-methoxy-*N*'-methylamide (5.05 g, 18.4 mmol) in diethyl ether (20 mL). The resulting reaction mixture was stirred for 30 min. After this period, the reaction was quenched with 10% NaHSO₄ solution (30 mL). The resulting reaction mixture was then warmed to 23°C and stirred at that temperature for 30 min. The resulting solution was filtered and the filter cake was washed by two portions of diethyl ether. The combined organic layers were washed with saturated sodium bicarbonate, brine and dried over anhydrous MgSO₄. Evaporation of the solvent under reduced pressure afforded the title aldehyde 4 (3.41 g) as a pale yellow oil. The resulting aldehyde was used immediately without further purification. ¹H NMR (400 MHz, CDCl₃) δ 9.5 (s, 1H), 4.9 (s, 1H), 4.2 (broad m, 1H), 1.8-1.6 (m, 2H), 1.44 (s, 9H), 1.49-1.39 (m, 1H), 0.96 (dd, 6H, J = 2.7, 6.5 Hz).

Ethyl (4*S*,5*S*)- and (4*R*,5*S*)-5-[(*tert*-Butoxycarbonyl)amino]-4-hydroxy-7-methyloct-2-ynoate (5).

To a stirred solution of diisopropylamine (1.1 mL, 7.9 mmol) in dry THF (60 mL) at 0°C under N₂ atmosphere, was added *n*-BuLi (1.6 M in hexane, 4.95 mL, 7.9 mmol) dropwise. The resulting solution was stirred at 0°C for 5 min and then warmed to 23°C and stirred for 15 min. The mixture was cooled to -78°C and ethyl propiolate (801 μL) in THF (2 mL) was added dropwise over a period of 5 min. The mixture was stirred for 30 min, after

which N-Boc-L-leucinal 4 (1.55 g, 7.2 mmol) in 8 mL of dry THF was added. The resulting mixture was stirred at -78°C for 1 h. After this period, the reaction was quenched with acetic acid (5 mL) in THF (20 mL). The reaction mixture was warmed up to 23°C and brine solution was added. The layers were separated and the organic layer was washed with saturated sodium bicarbonate and dried over Na₂SO₄. Evaporation of the solvent under reduced pressure provided a residue which was purified by flash silica gel chromatography (15 % ethyl acetate / hexane) to afford a mixture (3:1) of acetylenic alcohols 5 (0.96 g, 42 %). ¹H NMR (300 MHz, CDCl₃) δ 4.64 (d, 1H, J = 9.0 Hz), 4.44 (broad s, 1H), 4.18 (m, 2H), 3.76 (m, 1H), 1.63 (m, 1H), 1.43-1.31 (m, 2H), 1.39 (s, 9H), 1.29-1.18 (m, 3H), 0.89 (m, 6H). (5S,1'S)-5-[1'-[(tert-Butoxycarbonyl)amino]-3'-methylbutyl]-dihydrofuran-2(3H)-one (7).

To a stirred solution of the above mixture of acetylenic alcohols (1.73 g, 5.5 mmol) in ethyl acetate (20 mL) was added 5% Pd/BaSO₄ (1 g). The resulting mixture was hydrogenated at 50 psi for 1.5 h. After this period, the catalyst was filtered off through a plug of Celite and the filtrate was concentrated under reduced pressure. The residue was dissolved in toluene (20 mL) and acetic acid (100 µL). The reaction mixture was refluxed for 6 h. After this period, the reaction was cooled to 23°C and the solvent was evaporated to give a residue which was purified by flash silica gel chromatography (40% diethyl ether / hexane) to yield the (5S, 1S')-gamma-lactone 7 (0.94 g, 62.8 % and the (5R, 1S'')-gamma-lactone 6 (0.16 g, 10.7 %). Lactone 7: ¹H NMR (400 MHz, CDCl₃) δ 4.50-4.44 (m, 2H), 3.84-3.82 (m, 1H), 2.50 (t, 2H, J = 7.8 Hz), 2.22-2.10 (m, 2H), 1.64-1.31 (m, 3H), 1.41 (s, 9H), 0.91 (dd, 6H, J = 2.2, 6.7 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 177.2, 156.0, 82.5, 79.8, 51.0, 42.2, 28.6, 28.2, 24.7, 24.2, 23.0, 21.9. (3R,5S,1'S)-5-[1'-[(tert-Butoxycarbonyl)amino]-3'-methylbutyl]-3-methyl dihydrofuran-2(3H)-one (8).

To a stirred solution of the lactone 7 (451.8 mg, 1.67 mmol) in dry THF (8 mL) at -78°C under N₂ atmosphere, was added lithium hexamethyldisilazane (3.67 mL, 1.0 M in THF) over a period of 3 min. The

5 resulting mixture was stirred at -78°C for 30 min to generate the lithium enolate. After this period, MeI (228 μL) was added dropwise and the resulting mixture was stirred at -78°C for 20 min. The reaction was
10 quenched with saturated aqueous NH_4Cl solution and was allowed to warm to 23°C . The reaction mixture was concentrated under reduced pressure and the residue was extracted with ethyl acetate (3 x 100 mL). The combined organic layers were washed with brine and dried over anhydrous Na_2SO_4 .
15 Evaporation of the solvent afforded a residue which was purified by silica gel chromatography (15 % ethyl acetate / hexane) to furnish the alkylated lactone 8 (0.36 g, 76 %) as an amorphous solid. ^1H NMR (300 MHz, CDCl_3) δ 4.43 (broad t, 1H, J = 6.3 Hz), 4.33 (d, 1H, J = 9.6 Hz), 3.78 (m, 1H), 2.62 (m, 1H), 2.35 (m, 1H), 1.86 (m, 1H), 1.63-1.24 (m, 3H), 1.37 (s, 9H), 1.21 (d, 3H, J = 7.5 Hz), 0.87 (dd, 6H, J = 2.6, 6.7 Hz); ^{13}C NMR (75 MHz, CDCl_3) δ 180.4, 156.0, 80.3, 79.8, 51.6, 41.9, 34.3, 32.5, 28.3, 24.7, 23.0, 21.8, 16.6.

(2R,4S,5S)-5-[(tert-Butoxycarbonyl)amino]-4-[(tert-butylidimethylsilyl)oxy]-2,7-dimethyloctanoic acid (9).

To a stirred solution of lactone 8 (0.33 g, 1.17 mmol) in THF (2 mL) was added 1 N aqueous LiOH solution (5.8 mL). The resulting mixture was stirred at 23°C for 10 h. After this period, the reaction mixture was concentrated under reduced pressure and the remaining aqueous residue was cooled to 0°C and acidified with 25% citric acid solution to pH 4. The resulting acidic solution was extracted with ethyl acetate (3 x 50 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated to yield the corresponding hydroxy acid (330 mg) as a white foam. This hydroxy acid was used directly for the next reaction without further purification.

To the above hydroxy acid (330 mg, 1.1 mmol) in anhydrous DMF was added imidazole (1.59 g, 23.34 mmol) and tert-butylidimethylchlorosilane (1.76 g, 11.67 mmol). The resulting mixture was stirred at 23°C for 24 h. After this period, MeOH (4 mL) was added and the mixture was stirred for 1 h. The mixture was diluted with 25% citric acid (20

5 mL) and was extracted with ethyl acetate (3 x 20 mL). The combined
extracts were washed with water, brine and dried over anhydrous Na₂SO₄.
Evaporation of the solvent gave a viscous oil which was purified by flash
chromatography over silica gel (35% ethyl acetate / hexane) to afford the
10 silyl protected acid 9 (0.44 g, 90 %). IR (neat) 3300-3000 (broad), 2955,
2932, 2859, 1711 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆, 343 K) delta 6.20
(broad s, 1 H), 3.68 (m, 1H), 3.51 (broad s, 1H), 2.49-2.42 (m, 1H), 1.83 (t,
1H, J = 10.1 Hz), 1.56 (m, 1H), 1.37 (s, 9H), 1.28-1.12 (m, 3H), 1.08 (d, 3H,
15 J = 7.1 Hz), 0.87 (d, 3H, J = 6.1 Hz), 0.86 (s, 9 H), 0.82 (d, 3H, J = 6.5 Hz),
0.084 (s, 3H), 0.052 (s, 3H).
(2R,4S,5S)-5-[(fluorenylmethoxycarbonyl)amino]-4-[(tert-butyl-di-methyl
20 silyl)oxy]-2,7-dimethyloctanoic acid (10).

To a stirred solution of the acid 9 (0.17 g, 0.41 mmol) in
dichloromethane (2 mL) at 0°C was added trifluoroacetic acid (500 µL). The
resulting mixture was stirred at 0°C for 1 h and an additional portion (500
25 µL) of trifluoroacetic acid was added to the reaction mixture. The mixture
was stirred for an additional 30 min and the progress of the reaction was
monitored by TLC. After this period, the solvents were carefully removed
under reduced pressure at a bath temperature not exceeding 5°C. The residue
was dissolved in dioxane (3 mL) and NaHCO₃ (300 mg) in 5 mL of H₂O. To
30 this solution was added Fmoc-succinimide (166.5 mg, 0.49 mmol) in 5 mL
of dioxane. The resulting mixture was stirred at 23°C for 8 h. The mixture
was then diluted with H₂O (5 mL) and acidified with 25% aqueous citric acid
to pH 4. The acidic solution was extracted with ethyl acetate (3 x 50 mL).
The combined extracts were washed with brine, dried over Na₂SO₄ and
concentrated under reduced pressure to give a viscous oil residue.
40 Purification of the residue by flash chromatography over silica gel afforded
the Fmoc-protected acid 10 (137 mg, 61%) as a white foam. ¹H NMR (400
MHz, DMSO-d₆, 343 K) δ 7.84 (d, 2H, J = 7.4 Hz), 7.66 (d, 2H, J = 8 Hz),
7.39 (t, 2H, J = 7.4 Hz), 7.29 (m, 2H), 6.8 (s, 1H), 4.29-4.19 (m, 3H), 3.74-
45 3.59 (m, 2H), 2.49 (m, 1H), 1.88 (m, 1H), 1.58 (m, 1H), 1.31-1.17 (m, 3H).

5 1.10 (d, 3H, J = 7.1 Hz), 0.88 (s, 9H), 0.82 (d, 6H, J = 6.2 Hz), 0.089 (s, 3 H), 0.057 (s, 3H).

10 The synthesis of OM99-1 and OM99-2 were accomplished using solid state peptide synthesis procedure in which Leu*Ala was incorporated in the fourth step. The synthesized inhibitors were purified by reverse phase HPLC and their structure confirmed by mass spectrometry.

Example 8. Inhibition of Memapsin 2 by OM99-1 and OM99-2.

15 Enzyme activity was measured as described above, but with the addition of either OM99-1 or OM99-2. OM99-1 inhibited recombinant memapsin 2 as shown in Figure 5A. The K_i calculated is 3×10^{-8} M. The substrate used was a synthetic fluorogenic peptide substrate. The inhibition of OM99-2 on recombinant memapsin 2 was measured using the same fluorogenic substrate. The K_i value was determined to be 9.58×10^{-9} M. as shown in Figure 5B.

25 These results demonstrate that the predicted subsite specificity is accurate and that inhibitors can be designed based on the predicted specificity.

30 The residues in P1 and P1' are very important since the M2 inhibitor must penetrate the blood-brain barrier (BBB). The choice of Ala in P1' facilitates the penetration of BBB. Analogues of Ala side chains will also work. For example, in addition to the methyl side chain of Ala, substituted methyl groups and groups about the same size like methyl or ethyl groups can be substituted for the Ala side chain. Leu at P1 can also be substituted by groups of similar sizes or with substitutions on Leu side chain. For penetrating the BBB, it is desirable to make the inhibitors smaller. One can therefore use OM99-1 as a starting point and discard the outside subsites P4, P3, P3' and P4'. The retained structure Asn-Leu*Ala-Ala (SEQ ID NO:29) is then further evolved with substitutions for a tight-binding M2 inhibitor which can also penetrate the BBB.

45 Example 9. Crystallization and X-ray diffraction study of the protease domain of human memapsin 2 complexed to a specifically designed inhibitor, OM99-2.

5 The crystallization condition and preliminary x-ray diffraction data
on recombinant human memapsin 2 complexed to OM99-2 were determined.

Production of Recombinant Memapsin 2

10 About 50 mg of recombinant memapsin 2 was purified as described
in Example 3. For optimal crystal growth, memapsin 2 must be highly
purified. Memapsin 2 was over-expressed from vector pET11a-M2pd. This
memapsin 2 is the zymogen domain which includes the pro and catalytic
15 domains to the end of the C-terminal extension but does not include the
transmembrane and the intracellular domains. The vector was transfected
into E. coli BL21 (DE3) and plated onto ZB agar containing 50 mg/liter
ampicillin. A single colony was picked to inoculate 100 ml of liquid ZB
20 containing 5 mg ampicillin and cultured at 30 °C. for 18 hours, with shaking
at 220 RPM. Aliquots of approximately 15 ml of the overnight culture were
used to inoculate each 1 liter of LB containing 50 mg of ampicillin. Cultures
were grown at 37 °C. with shaking at 180 RPM, until an optical density at
25 600 nm near 0.8 was attained. At that time, expression was induced by
addition of 119 mg of IPTG to each liter of culture. Incubation was
continued for 3 additional hours post-induction.

30 Bacteria were harvested, suspended in 50 mM Tris, 150 mM NaCl,
pH 7.5 (TN buffer), and lysed by incubation with 6 mg lysozyme for 30
minutes, followed by freezing for 18 hours at -20 °C. Lysate was thawed and
made to 1 mM MgCl₂ then 1000 Kunitz units of DNase were added with
35 stirring, and incubated for 30 min. Volume was expanded to 500 ml with TN
containing 0.1 % Triton X-100 (TNT buffer) and lysate stirred for 30
minutes. Insoluble inclusion bodies containing greater than 90% memapsin
2 protein were pelleted by centrifugation, and washed by resuspension in
40 TNT with stirring for 1-2 hours. Following three additional TNT washes, the
memapsin 2 inclusion bodies were dissolved in 40 ml of 8 M urea, 1 mM
EDTA, 1 mM glycine, 100 mM Tris base, 100 mM beta-mercaptoethanol (8
45 M urea buffer). Optical density at 280 nm was measured, and volume
expanded with 8 M urea buffer to achieve final O.D. near 0.5, with addition
of sufficient quantity of beta-mercaptoethanol to attain 10 mM total, and 10

5 mM DTT, 1 mM reduced glutathione, 0.1 mM oxidized glutathione. The pH of the solution was adjusted to 10.0 or greater, and divided into four aliquots of 200 ml each. Each 200 ml was rapidly-diluted into 4 liters of 20 mM Tris base, with rapid stirring. The pH was adjusted immediately to 9.0, with 1 M HCl, and stored at 4 °C overnight. The following morning the diluted memapsin 2 solution was maintained at room temperature for 4-6 hours followed by adjusting pH to 8.5 and replacing the flasks to the 4 °C room. The same procedure was followed the next day with adjustment of pH to 8.0.

This memapsin 2 solution was allowed to stand at 4 °C for 2-3 weeks. The total volume of approximately 16 liters was concentrated to 40 mls using ultra-filtration (Millipore) and stir-cells (Amicon), and centrifuged at 140,000 xg at 30 minutes in a rotor pre-equilibrated to 4 °C. The recovered supernatant was applied to a 2.5 x 100 cm column of S-300 equilibrated in 0.4 M urea, 20 mM Tris-HCl, pH 8.0, and eluted with the same buffer at 30 ml/hour. The active fraction of memapsin 2 was pooled and further purified in a FPLC using a 1 ml Resource-Q (Pharmacia) column. Sample was filtered, and applied to the Resource-Q column equilibrated in 0.4 M urea, 50 mM Tris-HCl, pH 8.0. Sample was eluted with a gradient of 0 - 1 M NaCl in the same buffer, over 30 ml at 2 ml/min. The eluents containing memapsin 2 appeared near 0.4 M NaCl which was pooled for crystallization procedure at a concentration near 5 mg/ml.

The amino-terminal sequence of the protein before crystallization showed two sequences starting respectively at residues 28p and 30p. Apparently, the pro peptide of recombinant pro-memapsin 2 had been cleaved during the preparation by a yet unidentified proteolytic activity.

The activation of the folded pro-enzyme to mature enzyme, memapsin 2, was carried out as described above, i.e., incubation in 0.1 M sodium acetate pH 4.0 for 16 hours at 22 °C. Activated enzyme was further purified using anion-exchange column chromatography on Resource-Q anion exchange column. The purity of the enzyme was demonstrated by SDS-gel electrophoresis. At each step of the purification, the specific activity of the enzyme was assayed as described above to ensure the activity of the enzyme.

Preliminary Crystallization with OM99-2

Crystal trials were performed on purified memapsin 2 in complex with a substrate based transition-state inhibitor OM99-2 with a $K_i = 10$ nM. OM99-2 is equivalent to eight amino-acid residues (including subsites S4, S3, S2, S1 S1', S2', S3' and S4' in a sequence FVNLADEF) with the substitution of the peptide bond between the S1 and S1' (L-A) by a transition-state isostere hydroxyethylene. Purified M2 was concentrated and mixed with 10 fold excessive molar amount of inhibitor. The mixture was incubated at room temperature for 2-3 hours to optimize the inhibitor binding. The crystallization trial was conducted at 20 °C using the hanging drop vapor diffusion procedure. A systemic search with various crystallization conditions was conducted to find the optimum crystallization conditions for memapsin 2/OM99-2 inhibitor complex. For the first step, a coarse screen aimed at covering a wide range of potential conditions were carried out using the Sparse Matrix Crystallization Screen Kits purchased from Hampton Research. Protein concentration and temperature were used as additional variables. Conditions giving promising (micro) crystals were subsequently used as starting points for optimization, using fine grids of pH, precipitants concentration etc.

Crystals of memapsin-inhibitor complex were obtained at 30% PEG 8000, 0.1 M NaCocadylate, pH 6.4. SDS gel electrophoresis of a dissolved crystal verified that the content of the crystal to be memapsin 2. Several single crystals (with the sizes about 0.3 mm x 0.2 mm x 0.1 mm) were carefully removed from the cluster for data collection on a Raxis IV image plate. These results showed that the crystals diffract to 2.6 Å. A typical protein diffraction pattern is shown in Figure 6. An X-ray image visualization and integration software—Denzo, was used to visualize and index the diffraction data. Denzo identified that the primitive orthorhombic lattice has the highest symmetry with a significantly low distortion index. The unit cell parameters were determined as: $a=89.1$ Å, $b=96.6$ Å, $c=134.1$ Å, $\alpha=\beta=\gamma=90^\circ$. There are two memapsin 2/OM99-2 complexes per

5 crystallographic asymmetric unit, the V_m of the crystal is $2.9 \text{ \AA}^3/\text{Da}$.
Diffraction extinctions suggested that the space group is $P2_12_12_1$.

10 With diffraction of the current crystal to 2.6 \AA , the crystal structure
obtained from these data has the potential to reach atomic solution, i.e., the
three-dimensional positions of atoms and chemical bonds in the inhibitor
and in memapsin 2 can be deduced. Since memapsin 2 sequence is
15 homologous with other mammalian aspartic proteases, e.g., pepsin or
cathepsin D, it is predicted that the three dimensional structures of
memapsin 2 will be similar (but not identical) to their structures. Therefore,
in the determination of x-ray structure from the diffraction data obtained
from the current crystal, it is likely the solution of the phase can be obtained
20 from the molecular replacement method using the known crystal structure
of aspartic proteases as the search model.

Further Crystallization Studies

25 Concentrated memapsin 2 was mixed with 10-fold molar excessive
of the inhibitor. The mixture was incubated at room temperature for 2-3
hours to optimize inhibitor binding, and then clarified with a 0.2 micron
filter using centrifugation. Crystals of memapsin 2-inhibitor complex were
30 grown at 20°C by hanging drop vapor diffusion method using equal
volumes of enzyme-inhibitor and well solution. Crystals of quality suitable
for diffraction studies were obtained in two weeks in 0.1 M sodium
cacodylate, pH 7.4, 0.2 M $(\text{NH}_4)_2\text{SO}_4$, and 22.5% PEG8000. The typical
35 size of the crystals was about $0.4 \times 0.4 \times 0.2 \text{ mm}^3$.

Diffraction data were measured on a Raxis-IV image plate with a
Rigaku X-ray generator, processed with the HKL program package [Z.
40 Otwinowski, W. Minor, Methods Enzymol. 276, 307 (1997)] A single
crystal of approximately $0.4 \times 0.4 \times 0.2 \text{ mm}^3$ in size was treated with a cryo-
protection solution of 25% PEG8000, 20% glycerol, 0.1 M sodium-
cacodylate pH 6.6, and 0.2 M $(\text{NH}_4)_2\text{SO}_4$ and then flash-cooled with liquid
45 nitrogen to about -180°C for data collection. Diffraction was observed to
at least 1.9 \AA . The crystal form belongs to space group $P2_1$ with two

5 memapsin 2/OM99-2 complexes per crystallographic asymmetric unit and
56% solvent content.

10 Molecular replacement was performed with data in the range of
15.0-3.5 Å using program AmoRe. CCP4 package [Navaza, J., *Acta
Crystallog. Sect. A*, 50, 157 (1994)]. Pepsin, a human aspartic protease
with 22% sequence identity, was used as the search model (PDB id 1psn).
Rotation and translation search, followed by rigid body refinement,
15 identified a top solution and positioned both molecules in the asymmetric
unit. The initial solution had a correlation coefficient of 22% and an R-
factor of 0.51. The refinement was carried out using the program CNS
[Brunger et al., *Acta Crystallogr. Sect. D*, 54, 905 (1998)]. 10% of
20 reflections were randomly selected prior to refinement for R_{free} monitoring
[Bruger, A.T., X-PLOR Version 3.1: A system for X-ray Crystallography
and NMR. Yale University Press, New Haven, CT (1992)]. Molecular
25 graphics program [Jones, T.A., et al., Improved methods for building
protein models in electron density maps and location of errors in these
models. *Acta Crystallogr. Sect. A* 47, 110 (1991)] was used for map display
and model building. From the initial pepsin model, corresponding amino
30 acid residues were changed to that of memapsin 2 according to sequence
alignment. The side chain conformations were decided by the initial
electron density map and a rotamer library. This model was refined using
molecular dynamics and energy minimization function of CNS [Bruger,
35 A.T., et al., *Acta Crystallogr. Sect. D*, 54, 905 (1998)]. The first cycle of
refinement dropped the R_{working} to 41% and the R_{free} to 45%. At this stage,
electron densities in the omit map clearly showed the inhibitor configuration
40 in the active site cleft. Structural features unique to memapsin 2 in chain
tracing, secondary structure, insertions, deletions and extensions (as
compared to the search model) are identified and constructed in subsequent
iterations of crystallographic refinement and map fitting. The inhibitor was
45 built into the corresponding electron density.

About 440 solvent molecules were then gradually added to the
structure as identified in the $|F_o|-|F_c|$ map contoured at the 3 sigma level.

Non-crystallographic symmetry restriction and averaging were used in early stages of refinement and model building. Bulk solvent and anisotropic over-all B factor corrections were applied through the refinement. The final structure was validated by the program PROCHECK Laskowski, R.A. et al., J. Appl. Crystallog. 26, 283 (1993) which showed that 95% of the residues are located in the most favored region of the Ramachandran plot. All the main chain and side chain parameters are within or better than the standard criteria. The final R_{working} and R_{free} are 18% and 22% respectively. Refinement statistics are listed in Table 2.

Table 2. Data Collection and Refinement Statistics

A. Data Statistics

Space group	P2 ₁
Unit Cell (a, b, and c in Å)	53.7, 85.9, 109.2
(α , β and γ in degrees)	90.0, 101.4, 90.0
Resolution (Å)	25.0-1.9
Number of observed reflections	144,164
Number of unique reflections	69,056
R_{merge}^a	0.061 (0.25)
Data completeness (%) (25.0-1.9 Å)	90.0 (68.5)
$\langle I/\sigma(I) \rangle$	13.7 (3.0)

B. Refinement Statistics

R_{working}^b	0.186
R_{free}^b	0.228
RMS deviation from ideal values	
Bond length (Å)	0.014
Bond angle (Deg)	1.7
Number of water molecules	445
Average B-factor (Å ²)	
Protein	28.5
Solvent	32.2

^a $R_{\text{merge}} = \sum_{hkl} \sum_i |I_{hkl,i} - \langle I_{hkl} \rangle| / \sum_{hkl} \langle I_{hkl} \rangle$, where $I_{hkl,i}$ is the intensity of the i th measurement and $\langle I_{hkl} \rangle$ is the weighted mean of all measurements of I_{hkl} .

^b $R_{\text{working (free)}} = \sum |F_o| - |F_c| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factors. Numbers in parentheses are the corresponding numbers for the highest resolution shell (2.00-1.9 Å). Reflections with $F_o/\sigma(F_o) \geq 0.0$ are included in the refinement and R factor calculation.

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Memapsin 2 Crystal Structure.

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The bilobal structure of memapsin 2 (Figure 7) is characteristic of aspartic proteases (Tang, J., et al., Nature 271, 618-621 (1978)) with the conserved folding of the globular core. The substrate binding cleft, where the inhibitor is bound (Figure 7), is located between the two lobes. A pseudo two-fold symmetry between the N- (residues 1-180) and C- (residues 181-385) lobes (Figure 7), which share 61 superimposable atoms with an overall 2.3 Å rms deviation using a 4 Å cutoff. The corresponding numbers for pepsin are 67 atoms and 2.2 Å. Active-site Asp³² and Asp²²⁸ and the surrounding hydrogen-bond network are located in the center of the cleft (Figure 7) and are conserved with the typical active-site conformation (Davies, D. R., Annu. Rev. Biophys. Chem. 19, 189 (1990)). The active site carboxyls are, however, not co-planar and the degree of which (50°) exceeds those observed previously.

Compared to pepsin, the conformation of the N-lobe is essentially conserved (Sielecki et al., 1990). The most significant structural differences are the insertions and a C-terminal extension in the C-lobe. Four insertions in helices and loops (Figure 7) are located on the adjacent molecular surface. Insertion F, which contains four acidic residues, is the most negatively charged surface on the molecule. Together, these insertions enlarged significantly the molecular boundary of memapsin 2 as compared to pepsin (Figure 8). These surface structural changes may have function in the association of memapsin 2 with other cell surface components. Insertions B and E are located on the other side of the molecule (Figure 7). The latter contains a beta-strand that paired with part of the C-terminal extension G. A six-residue deletion occurs at position 329 on a loop facing the flap on the opposite side of the active-site cleft, resulting in an apparently more accessible cleft. Most of the C-terminal extension (residues 359-393) is in highly ordered structure. Residues 369-376 form a beta structure with 7 hydrogen bonds to strand 293-299, while residues 378-383 form a helix (Figures 7 and 8). Two disulfide pairs (residues 155/359 and 217/382) unique to memapsin 2 fasten both ends of the extension region to

5 the C-lobe. This C-terminal extension is much longer than those observed
previously and is conformationally different [Cutfield, S. M., et al.,
Structure 3, 1261 (1995); Abad-Zapatero, C., et al., Protein Sci. 5, 640
10 (1996); Symersky, J. et al., Biochemistry 36, 12700 (1997); Yang, J., et al.,
Acta Crystallogr. D 55, 625 (1999)]. The last eight residues (386-393) are
not seen in the electron density map; they may form a connecting stem
between the globular catalytic domain and the membrane anchoring
domain.

15 Of the 21 putative pro residues only the last six, 43p-48p, are visible
in the electron density map. The remainders are likely mobile. Pro-
memapsin expressed in mammalian cell culture has an N-terminus position
20 at Glu^{33p}. However, an Arg-Arg sequence present at residues 43p-44p is a
frequent signal for pro-protein processing, e.g., in prorenin (Corvol, P. et al.,
Hypertension 5, 13-9 (1983)). Recombinant memapsin 2 derived from this
cleavage is fully active. The mobility of residues 28p-42p suggests that they
25 are not part of the structure of mature memapsin 2.

Memapsin 2-OM99-2 Interaction.

30 The binding of the eight-residue inhibitor OM99-2 in the active-site
cleft shares some structural features with other aspartic protease-inhibitor
complexes [Davies, D.R., Annu. Rev. Biophys. Chem. 19, 189 (1990);
Bailey and Cooper, (1994); Dealwis et al., (1994)]. These include four
35 hydrogen bonds between the two active-site aspartics to the hydroxyl of the
transition-state isostere, the covering of the flap (residues 69-75) over the
central part of the inhibitor and ten hydrogen bonds to inhibitor backbone
(Figure 9). Most of the latter are highly conserved among aspartic proteases
40 [Davies, D. R. Annu. Rev. Biophys. Chem. 19, 189 (1990); Bailey and
Cooper, (1994); Dealwis et al., (1994)] except that hydrogen bonds to Gly¹¹
and Tyr¹⁹⁸ are unique to memapsin 2. These observations illustrate that the
manner by which memapsin 2 transition-state template for substrate peptide
45 backbone and mechanism of catalysis are similar to other aspartic proteases.
These common features are, however, not the decisive factors in the design
of specific memapsin 2 inhibitors with high selectivity.

5 The observation important for the design of inhibitor drugs is that
the memapsin 2 residues in contact with individual inhibitor side chains
(Figure 9) are quite different from those for other aspartic proteases. These
10 side chain contacts are important for the design of tight binding inhibitor
with high selectivity. Five N-terminal residues of OM99-2 are in extended
conformation and, with the exception of P₁' Ala, all have clearly defined
15 contacts (within 4 Å of an inhibitor side chain) with enzyme residues in the
active-site cleft (Figure 9).

 The protease S₄ subsite is mostly hydrophilic and open to solvent.
The position of inhibitor P₄ Glu side chain is defined by hydrogen bonds to
20 Gly¹¹ and to P₂ Asn (Figure 9) and the nearby sidechains of Arg²³⁵ and
Arg³⁰⁷, which explains why the absence of this residue from OM99-2 cause
a 10-fold increase in K_i. Likewise, the protease S₂ subsite is relatively
hydrophilic and open to solvent. Inhibitor P₂ Asn side chain has hydrogen
25 bonds to P₄ Glu and Arg²³⁵. The relatively small S₂ residues Ser³²⁵ and
Ser³²⁷ (Gln and Met respectively in pepsin) may fit a side chain larger than
Asn. Memapsin 2 S₁ and S₃ subsites, which consist mostly of hydrophobic
residues, have conformations very different from pepsin due to the deletion
30 of pepsin helix h_{H2} (DeCalwis, et al., (1994)). The inhibitor side chains of P₃
Val and P₁ Leu are closely packed against each other and have substantial
hydrophobic contacts with the enzyme (Figure 9), especially P₃ interacts
with Tyr⁷¹ and Phe¹⁰⁸. In the beta- secretase site of native APP, the P₂ and
35 P₁ residues are Lys and Met respectively. Swedish mutant APP has Asn and
Leu in these positions respectively, resulting in a 60-fold increase of k_{cat}/K_m
over that for native APP and an early onset of AD described by Mullan, M.,
et al. [Nat. Genet. 2, 340 (1992)]. The current structure suggests that
40 inhibitor P₂ Lys would place its positively charge in an unfavorable
interaction with Arg²³⁵ with a loss of hydrogen bond to Arg²³⁵, while P₁ Met
would have less favorable contact with memapsin 2 than does leucine in this
45 site (Figure 10). No close contact with memapsin 2 was seen for P₁' Ala and
an aspartic at this position, as in APP, may be accommodated by interacting
with Arg²²⁸.

5 The direction of inhibitor chain turns at P₂' and leads P₃' and P₄'
toward the protein surface (Figure 10). As a result, the side-chain position
of P₂' Ala deviates from the regular extended conformation. The side chains
of P₃' Glu and P₄' Phe are both pointed toward molecular surface with little
10 significant interaction with the protease (Figure 10). The relatively high B-
factors (58.2 Å² for Glu and 75.6 Å² for Phe) and less well-defined electron
density suggests that these two residues are relatively mobile, in contrast to
the defined structure of the S₃' and S₄' subsites in renin-inhibitor (CH-66)
15 complex (Dealwis et al., 1994). The topologically equivalent region of these
renin subsites (residues 292 - 297 in pepsin numbering) is deleted in
memapsin 2. These observations suggest that the conformation of three C-
terminal residues of OM99-2 may be a functional feature of memapsin 2,
20 possibly a way to lead a long protein substrate out of the active-site cleft.
Example 10: Using The Crystal Structure to Design Inhibitors.

Pharmaceutically acceptable inhibitor drugs normally post a size limit
25 under 800 daltons. In the case of memapsin 2 inhibitors, this requirement
may even be more stringent due to the need for the drugs to penetrate the
blood-brain barrier [Kearney and Aweeka, (1999)]. In the current model,
well defined subsite structures spending P₄ to P₂' provide sufficient template
30 areas for rational design of such drugs. The spacial relationships of
individual inhibitor side chain with the corresponding subsite of the enzyme
as revealed in this crystal structure permits the design of new inhibitor
structures in each of these positions. It is also possible to incorporate the
unique conformation of subsites P₂', P₃' and P₄' into the selectivity of
35 memapsin 2 inhibitors. The examples of inhibitor design based on the current
crystal structure are given below.

40 Example A: Since the side chains of P₃ Val and P₁ Leu are packed against
each other and there is no enzyme structure between them, cross-linking
these side chains would increase the binding strength of inhibitor to
memapsin 2. This is because when binding to the enzyme, the cross-linked
45 inhibitors would have less entropy difference between the free and bound
forms than their non-cross-linked counterparts [Khan, A.R., et al.,

5 Biochemistry, 37, 16839 (1998)]. Possible structures of the cross-linked side chains include those shown in Figure 11.

10 Example B: The same situation exists between the P4 Glu and P2 Asn. The current crystal structure shows that these side chains are already hydrogen bonded to each other so the cross linking between them would also derive binding benefit as described in the Example A. The cross-linked structures include those shown in Figure 12.

15 Example C: Based on the current crystal structure, the P1' Ala side chain may be extended to add new hydrophobic, Van der Waals and H-bond interactions. An example of such a design is diagramed in Figure 13.

20 Example D: Based on the current crystal structure, the polypeptide backbone in the region of P1, P2, and P3, and the side chain of P1-Leu can be bridged into rings by the addition of two atoms (A and B in Figure 14). Also, a methyl group can be added to the beta-carbon of the P1-Leu (Figure 14).

Claims

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5 We claim:

1. Purified recombinant catalytically active memapsin 2.
2. The memapsin 2 of claim 1 having the amino acid sequence of SEQ ID NO. 2 or the sequence present in a homologous species.
- 10 3. The memapsin 2 of claim 2 of human origin and having the amino acid sequence of SEQ ID NO. 2.
4. The memapsin 2 of claim 1 not including the transmembrane domain.
- 15 5. The memapsin 2 of claim 1 expressed in a bacteria.
6. The memapsin 2 of claim 1 cleaving SEVKM/DAEFR (SEQ ID NO:4) and SEVNL/DAEFR (SEQ ID NO:5) at pH 4.0 with k_{cat}/K_m of less than or equal to $39.9 \text{ s}^{-1}\text{M}^{-1}$ and less than or equal to k_{cat} , 2.45 s^{-1} , K_m , 1 mM; k_{cat}/K_m , $2450 \text{ s}^{-1}\text{M}^{-1}$, respectively.
- 20 7. A method for producing catalytically active recombinant memapsin 2 comprising refolding the recombinant memapsin 2 under conditions which dissociate and then slowly refold the enzyme into a catalytically active form.
8. The method of claim 7 wherein the memapsin 2 is first dissolved in 8 M urea solution including one or more reducing agents at a pH of greater than 8.0.
- 30 9. The method of claim 8 wherein the memapsin 2 is then diluted into an aqueous buffer like 20 mM-Tris, pH 9.0, the pH slowly adjusted to approximately 8 with 1 M HCl, and the solution maintained at low temperature for approximately 24 to 48 hours before proceeding with purification.
- 40 10. The method of claim 8 wherein the memapsin 2 is then rapidly mixed with an aqueous buffer like 20 mM-Tris, pH 9.0, containing oxidized and reduced glutathione, the process repeated, then the urea concentration decreased to approximately 0.4 M and the pH of the solution slowly adjusted to 8.0.
- 45 11. The method of claim 8 wherein the memapsin 2 is dissolved in 8 M urea, pH 10.0, then rapidly diluted into an aqueous buffer like 20 mM

5 Tris base, pH 9.0, and maintained at low temperature several hours.
maintained at room temperature for several hours, and then the process
repeated at decreasing pH.

10 12. A method of isolating inhibitors of cleavage by memapsin 2
comprising

adding to one or more potential inhibitors catalytically active
recombinant memapsin 2 cleaving SEVKM/DAEFR (SEQ ID NO:4) and
15 SEVNL/DAEFR (SEQ ID NO:5) at pH 4.0 with k_{cat}/K_m of less than or equal
to $39.9 \text{ s}^{-1}\text{M}^{-1}$ and less than or equal to k_{cat} , 2.45 s^{-1} , K_m , 1 mM; k_{cat}/K_m , 2450
 $\text{s}^{-1}\text{M}^{-1}$, respectively, and a substrate for memapsin 2, and

screening for decreased cleavage of the substrate by the inhibitors.

20 13. The method of claim 12 wherein the inhibitors are in a library
of small synthetic molecules.

14. The method of claim 12 wherein the inhibitors are compounds
25 selected from the group consisting of proteins and peptides.

15. The method of claim 12 wherein the recombinant memapsin 2
is expressed in genetically engineered cells and the inhibitors and substrate
are added to the cells.

30 16. The method of claim 15 wherein the inhibitors are
oligonucleotides preventing or decreasing expression of catalytically active
memapsin 2.

35 17. The method of claim 14 wherein the compounds are isosteres
of the memapsin 2 active site defined by the presence of two catalytic
aspartic residues and substrate binding cleft.

40 18. The method of claim 12 further comprising identifying the
inhibitors maximally decreasing cleavage of substrate by the memapsin 2.

19. A method for designing or obtaining inhibitors of catalytically
active memapsin 2 comprising modeling an inhibitor based on the
crystallization coordinates of memapsin 2 or parameters of Table 2.

45 20. The method of claim 19 comprising using a computer
program to model a compound to determine its binding to the memapsin 2

5 active site defined by the presence of two catalytic aspartic residues and substrate binding cleft.

10- 21. The method of claim 19 comprising using a computer program to design a compound which binds to the memapsin 2 active site defined by the presence of two catalytic aspartic residues and substrate binding cleft.

15 22. The method of claim 19 further comprising screening compounds which bind to the active site defined by the presence of two catalytic aspartic residues and substrate binding cleft for inhibition of memapsin 2 catalytic activity.

20 23. A data base comprising binding properties and chemical structures of compounds designed or screened by modeling an inhibitor based on the crystallization coordinates of memapsin 2 or parameters of Table 2.

25 24. A method of treating or preventing Alzheimer's disease comprising administering administering to a patient in need thereof an inhibitor of memapsin 2 which binds to the active site of the memapsin 2 defined by the presence of two catalytic aspartic residues and substrate binding cleft.

30 25. The method of claim 24 wherein the inhibitor has an K_i of less than or equal to 10^{-7} M.

35 26. The method of claim 24 wherein the inhibitor is selected from the group consisting of proteins, peptides, oligonucleotides, and small synthetic molecules.

40 27. The method of claim 24 wherein the inhibitor is modeled based on the crystallization coordinates of memapsin 2 or parameters of Table 2.

45 28. A crystallized memapsin 2 comprising the active site defined by the presence of two catalytic aspartic residues and substrate binding cleft.

50 29. The memapsin 2 of claim 28 not including the transmembrane and/or intracellular domains.

- 5 30. The memapsin 2 of claim 28 having the parameters defined in
Table 2.
31. The memapsin 2 of claim 28 having a diffraction to at least
3.5 Å or less.
- 10- 32. The memapsin 2 of claim 31 having a diffraction to at least 2
Å or less.
33. A method for treatment or preventing Alzheimer's disease
15 comprising immunizing an individual in need thereof with catalytically
active memapsin 2 cleaving SEVKM/DAEFR (SEQ ID NO:4) and
SEVNL/DAEFR (SEQ ID NO:5) at pH 4.0 with k_{cat}/K_m of less than or equal
20 to $39.9 \text{ s}^{-1}\text{M}^{-1}$ and less than or equal to k_{cat} , 2.45 s^{-1} , K_m , 1 mM; k_{cat}/K_m , 2450
 $\text{s}^{-1}\text{M}^{-1}$, respectively, to elicit an effective amount of antibodies to reduce
cleavage by endogenous mamapsin 2.

1v MASMTGGQQM GRGSMAGVLP AHGTQHGIRL PLRSLGGAP IGLRLPRETD
 36p EEPEEPGRRG SFVEMVDNI.R GKSGQGYVE MTVGSPQTL NILVDTGSSN
 3e FAVGAAPHFF LHRYRQRLS STYRDLRKGV YVPYTOGKWE GELGTDLVSI
 88 PHGPNVTVRA NIAAITESDK FFINGSNWEG ILGLAYAEIA RPDDSLPEFF
 138 DSLVKQTHVP NLFSLQLCGA GFPLNQSEVL ASVGSGMIIG GIDHSLYTGS
 188 LWYTPIRREW YVEVIIVRVE INGQDLKMDK KEYNYDKSIV DSGTTNLRLP
 238 KKVFEAAVKS IKAASSTEKF PDGFWLGEQL VCWQAGTTPW NIFPVISLYL
 288 MGEVTNQSFRT ITILPQQYLR PVEDVATSQD DCYKFAISQS STGTVMGAVI
 338 MEGFYVVFDR ARKRIGFAVS ACHVHDEFRT AAVEGPFVTL DMEDCGYNIP
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 438 LLK*

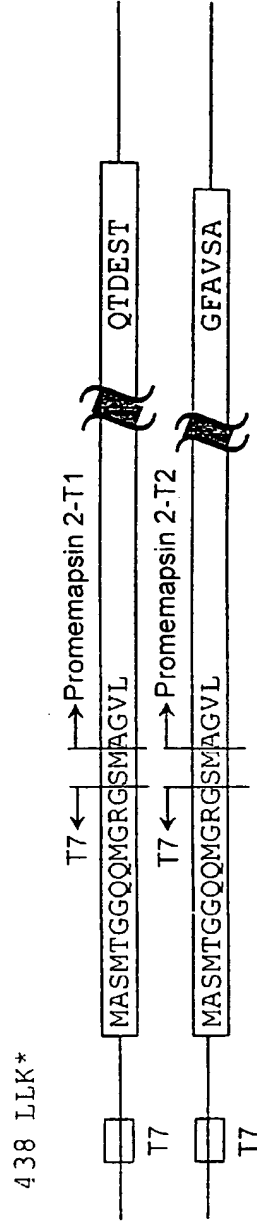


FIG. 1

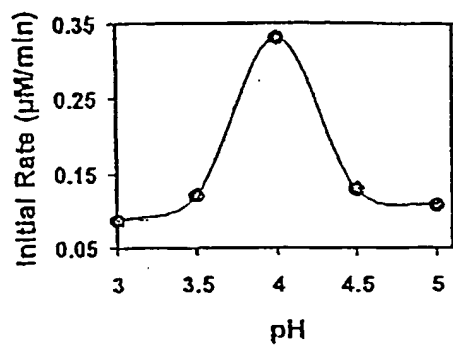


Fig. 2A

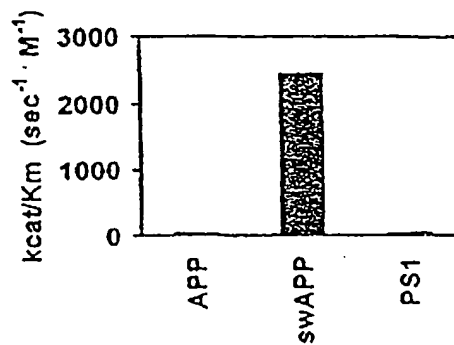
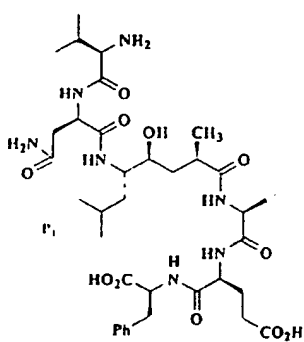
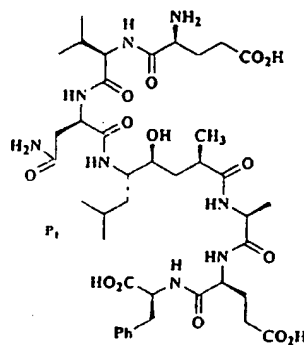


Fig. 2B



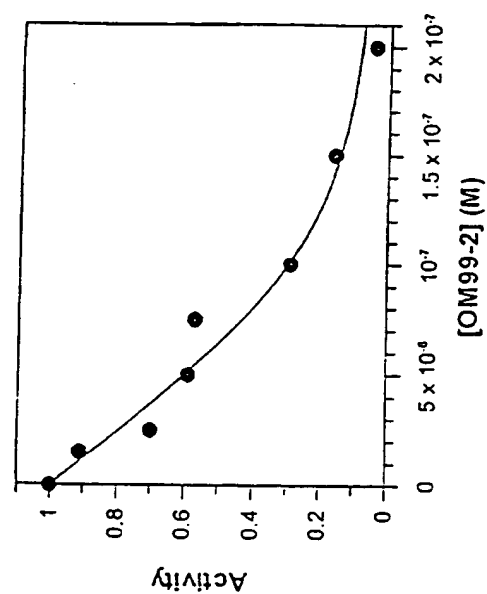
OM99-1

Fig. 3A



OM99-2

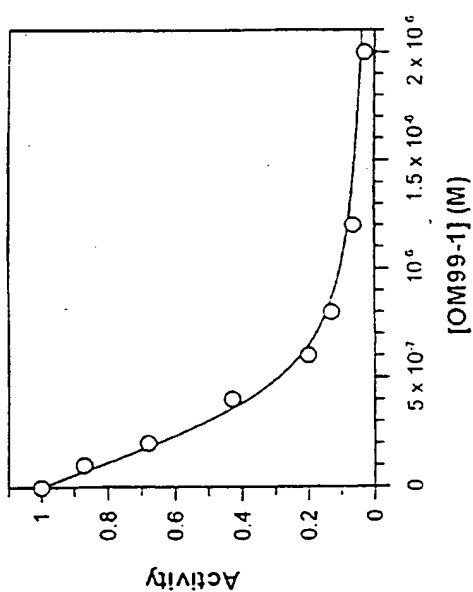
Fig. 3B



$[E]_0 = 0.11 \mu\text{M}$
 $[\text{Fluo Substrate}] = 29.9 \mu\text{M}$

Parameter	Value	Std. Error
Ki	9.58e-9	2.86e-10

Fig. 4B



$[\text{Mep2}] = 0.47 \mu\text{M}$
 $[\text{Fluo. Substrate}] = 29.9 \mu\text{M}$
 Buffer: Na Acetate 0.1 M, 5 % DMSO, pH 4.5 at 37°C
 Excitation at 350 nm
 Emission at 490 nm

Parameter	Value	Std. Error
Ki	6.84e-8	2.72e-9

Fig. 4A

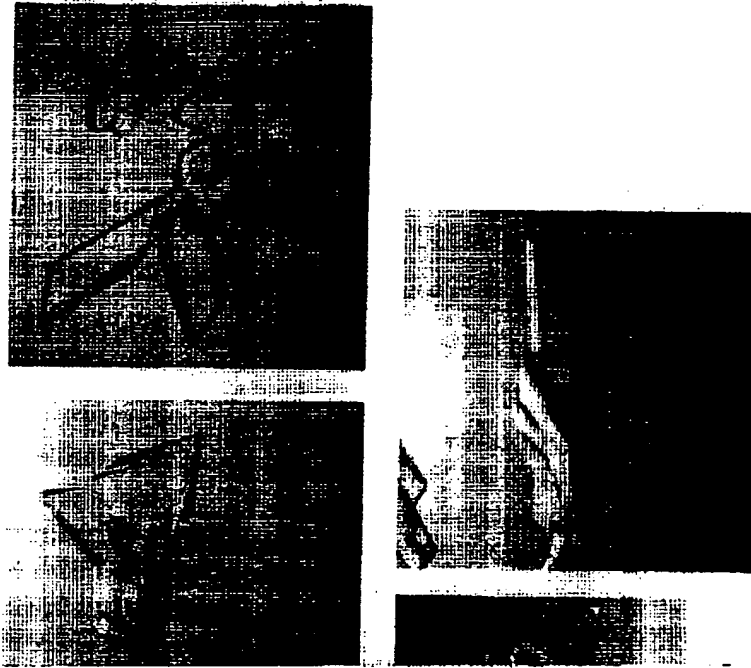


Fig. 5

Fig. 6

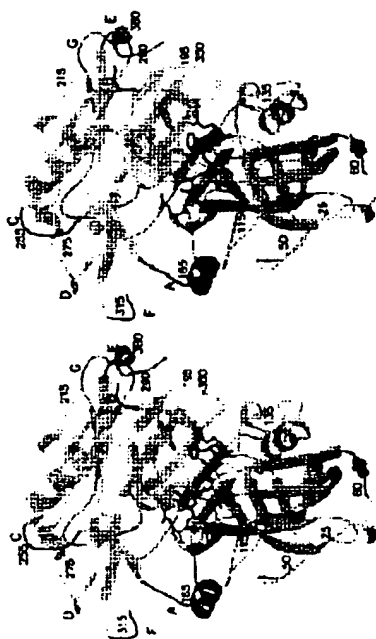
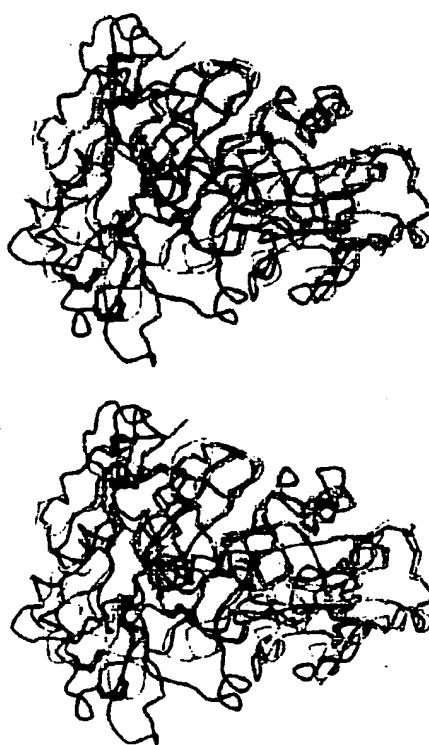


Fig. 7



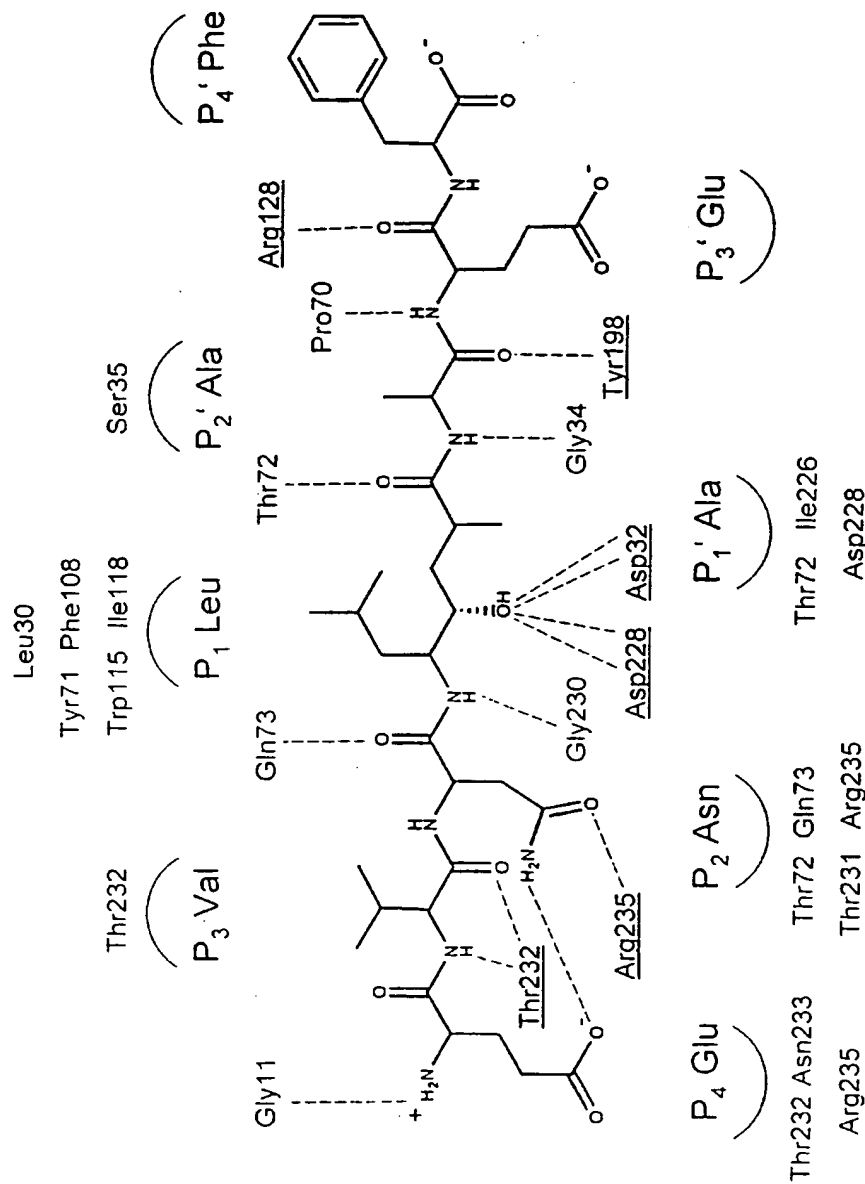
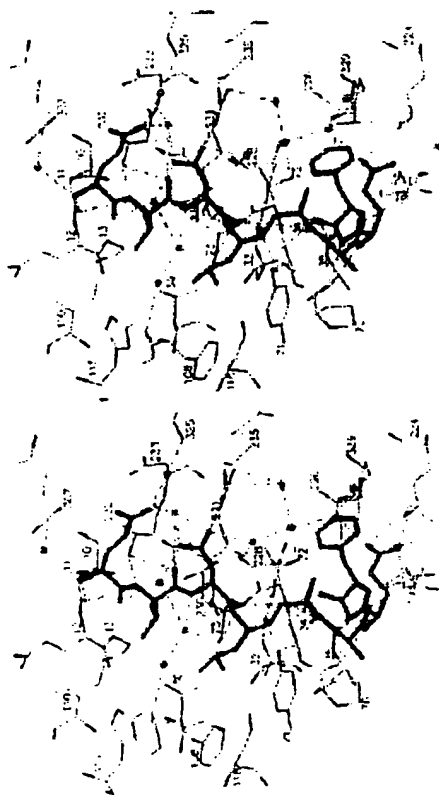


FIG. 8

Fig. 9



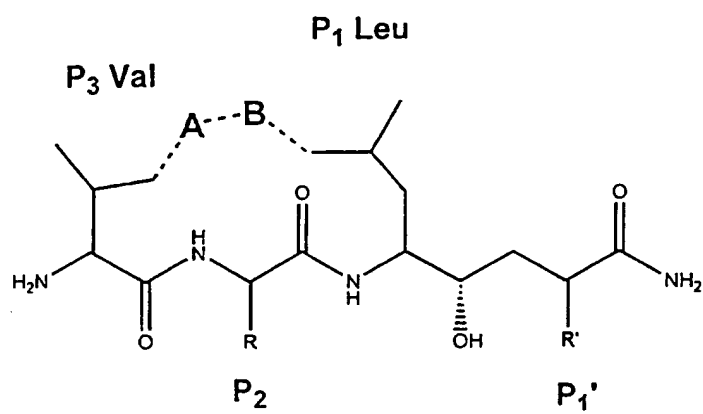


FIG. 10

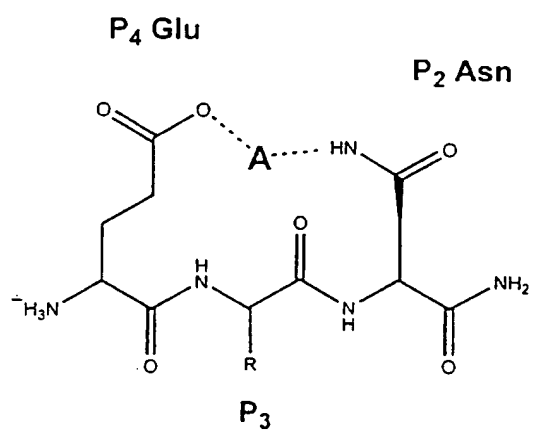


FIG. 11

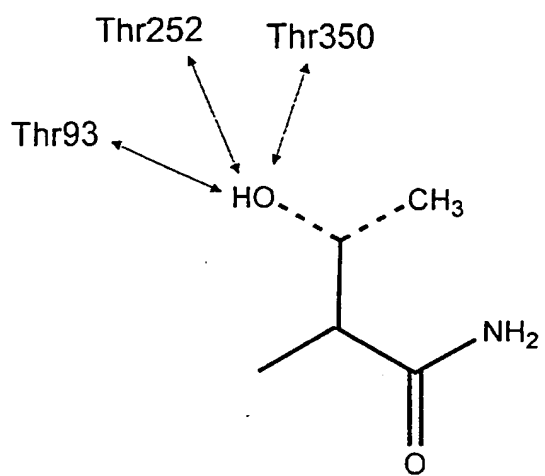


FIG. 12

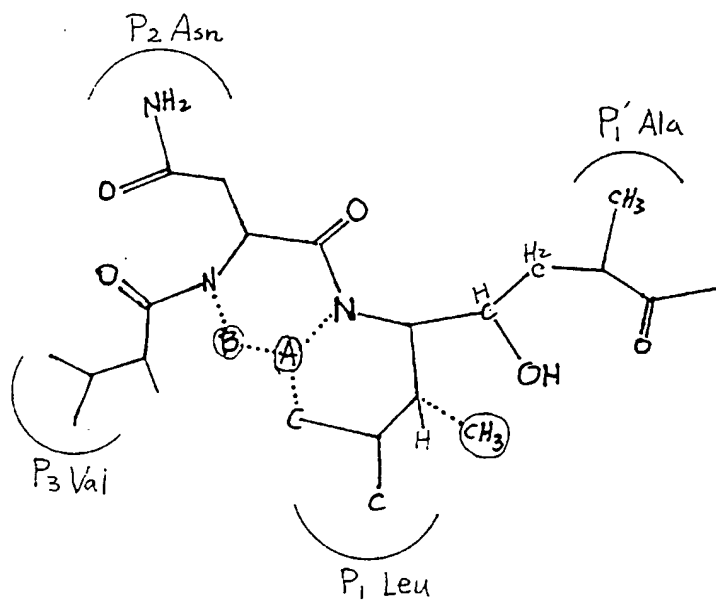


FIG. 13

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<223> Amino Acids 28-48 are remnant putative propeptide residues

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<223> Amino Acids 58-61, 78, 80, 82-83, 116, 118-121, 156, 166, 174, 246, 274, 276, 278-281, 283, and 376-377 are residues in contact with the OM99-2 inhibitor

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<223> Amino acids 54-57, 61-68, 73-80, 86-89, 109-111, 113-118, 123-134, 143-154, 165-168, 198-202, and 220-224 are N-lobe Beta Strands

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<223> Amino Acids 184-191 and 210-217 are N-lobe Helices

<220>

<223> Amino acids 237-240, 247-249, 251-256, 259-260, 273-275, 282-285, 316-318, 331-336, 342-348, 354-357, 366-370, 372-375, 380-383, 390-395, 400-405, and 418-420 are C-lobe Beta Strands

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<223> Amino Acids 286-299, 307-310, 350-353, 384-387, and 427-431 are C-lobe Helices

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35 40 45

Glu Met Val Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly Tyr Tyr Val
50 55 60

Glu Met Thr Val Gly Ser Pro Pro Gln Thr Leu Asn Ile Leu Val Asp

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Thr Gly Ser Ser Asn Phe Ala Val Gly Ala Ala Pro His Pro Phe Leu			
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His Arg Tyr Tyr Gln Arg Gln Leu Ser Ser Thr Tyr Arg Asp Leu Arg			
	100	105	110
Lys Gly Val Tyr Val Pro Tyr Thr Gln Gly Lys Trp Glu Gly Glu Leu			
	115	120	125
Gly Thr Asp Leu Val Ser Ile Pro His Gly Pro Asn Val Thr Val Arg			
	130	135	140
Ala Asn Ile Ala Ala Ile Thr Glu Ser Asp Lys Phe Phe Ile Asn Gly			
	145	150	155
Ser Asn Trp Glu Gly Ile Leu Gly Leu Ala Tyr Ala Glu Ile Ala Arg			
	165	170	175
Pro Asp Asp Ser Leu Glu Pro Phe Phe Asp Ser Leu Val Lys Gln Thr			
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His Val Pro Asn Leu Phe Ser Leu Gln Leu Cys Gly Ala Gly Phe Pro			
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Gly Gly Ile Asp His Ser Leu Tyr Thr Gly Ser Leu Trp Tyr Thr Pro			
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Ile Arg Arg Glu Trp Tyr Tyr Glu Val Ile Ile Val Arg Val Glu Ile			
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Asn Gly Gln Asp Leu Lys Met Asp Cys Lys Glu Tyr Asn Tyr Asp Lys			
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Ser Ile Val Asp Ser Gly Thr Thr Asn Leu Arg Leu Pro Lys Lys Val			
	275	280	285
Phe Glu Ala Ala Val Lys Ser Ile Lys Ala Ala Ser Ser Thr Glu Lys			
	290	295	300
Phe Pro Asp Gly Phe Trp Leu Gly Glu Gln Leu Val Cys Trp Gln Ala			
	305	310	315
Gly Thr Thr Pro Trp Asn Ile Phe Pro Val Ile Ser Leu Tyr Leu Met			

```

          325          330          335
Gly Glu Val Thr Asn Gln Ser Phe Arg Ile Thr Ile Leu Pro Gln Gln
          340          345          350
Tyr Leu Arg Pro Val Glu Asp Val Ala Thr Ser Gln Asp Asp Cys Tyr
          355          360          365
Lys Phe Ala Ile Ser Gln Ser Ser Thr Gly Thr Val Met Gly Ala Val
          370          375          380
Ile Met Glu Gly Phe Tyr Val Val Phe Asp Arg Ala Arg Lys Arg Ile
          385          390          395          400
Gly Phe Ala Val Ser Ala Cys His Val His Asp Glu Phe Arg Thr Ala
          405          410          415
Ala Val Glu Gly Pro Phe Val Thr Leu Asp Met Glu Asp Cys Gly Tyr
          420          425          430
Asn Ile Pro Gln Thr Asp Glu Ser Thr Leu Met Thr Ile Ala Tyr Val
          435          440          445
Met Ala Ala Ile Cys Ala Leu Phe Met Leu Pro Leu Cys Leu Met Val
          450          455          460
Cys Gln Trp Arg Cys Leu Arg Cys Leu Arg Gln Gln His Asp Asp Phe
          465          470          475          480
Ala Asp Asp Ile Ser Leu Leu Lys
          485

```

```

<210> 3
<211> 503
<212> PRT
<213> Homo sapiens

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```

<220>
<223> Pro-memapsin 2

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<220>
<223> Amino Acids 1-15 are vector-derived residues

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<220>
<223> Amino Acids 16-64 are a putative pro peptide

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<220>

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<223> Amino Acids 1-13 are the T7 promoter

<220>

<223> Amino Acids 16-456 are Pro-memapsin 2-T1

<220>

<223> Amino Acids 16-421 are Promemapsin 2-T2

<400> 3

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Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Gly Ser Met Ala
  1             5             10            15

Gly Val Leu Pro Ala His Gly Thr Gln His Gly Ile Arg Leu Pro Leu
      20             25             30

Arg Ser Gly Leu Gly Gly Ala Pro Leu Gly Leu Arg Leu Pro Arg Glu
      35             40             45

Thr Asp Glu Glu Pro Glu Glu Pro Gly Arg Arg Gly Ser Phe Val Glu
      50             55             60

Met Val Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly Tyr Tyr Val Glu
      65             70             75             80

Met Thr Val Gly Ser Pro Pro Gln Thr Leu Asn Ile Leu Val Asp Thr
      85             90             95

Gly Ser Ser Asn Phe Ala Val Gly Ala Ala Pro His Pro Phe Leu His
      100            105            110

Arg Tyr Tyr Gln Arg Gln Leu Ser Ser Thr Tyr Arg Asp Leu Arg Lys
      115            120            125

Gly Val Tyr Val Pro Tyr Thr Gln Gly Lys Trp Glu Gly Glu Leu Gly
      130            135            140

Thr Asp Leu Val Ser Ile Pro His Gly Pro Asn Val Thr Val Arg Ala
      145            150            155            160

Asn Ile Ala Ala Ile Thr Glu Ser Asp Lys Phe Phe Ile Asn Gly Ser
      165            170            175

Asn Trp Glu Gly Ile Leu Gly Leu Ala Tyr Ala Glu Ile Ala Arg Pro
      180            185            190

Asp Asp Ser Leu Glu Pro Phe Phe Asp Ser Leu Val Lys Gln Thr His
      195            200            205

```

Val Pro Asn Leu Phe Ser Leu Gln Leu Cys Gly Ala Gly Phe Pro Leu
 210 215 220
 Asn Gln Ser Glu Val Leu Ala Ser Val Gly Gly Ser Met Ile Ile Gly
 225 230 235 240
 Gly Ile Asp His Ser Leu Tyr Thr Gly Ser Leu Trp Tyr Thr Pro Ile
 245 250 255
 Arg Arg Glu Trp Tyr Tyr Glu Val Ile Ile Val Arg Val Glu Ile Asn
 260 265 270
 Gly Gln Asp Leu Lys Met Asp Cys Lys Glu Tyr Asn Tyr Asp Lys Ser
 275 280 285
 Ile Val Asp Ser Gly Thr Thr Asn Leu Arg Leu Pro Lys Lys Val Phe
 290 295 300
 Glu Ala Ala Val Lys Ser Ile Lys Ala Ala Ser Ser Thr Glu Lys Phe
 305 310 315 320
 Pro Asp Gly Phe Trp Leu Gly Glu Gln Leu Val Cys Trp Gln Ala Gly
 325 330 335
 Thr Thr Pro Trp Asn Ile Phe Pro Val Ile Ser Leu Tyr Leu Met Gly
 340 345 350
 Glu Val Thr Asn Gln Ser Phe Arg Ile Thr Ile Leu Pro Gln Gln Tyr
 355 360 365
 Leu Arg Pro Val Glu Asp Val Ala Thr Ser Gln Asp Asp Cys Tyr Lys
 370 375 380
 Phe Ala Ile Ser Gln Ser Ser Thr Gly Thr Val Met Gly Ala Val Ile
 385 390 395 400
 Met Glu Gly Phe Tyr Val Val Phe Asp Arg Ala Arg Lys Arg Ile Gly
 405 410 415
 Phe Ala Val Ser Ala Cys His Val His Asp Glu Phe Arg Thr Ala Ala
 420 425 430
 Val Glu Gly Pro Phe Val Thr Leu Asp Met Glu Asp Cys Gly Tyr Asn
 435 440 445
 Ile Pro Gln Thr Asp Glu Ser Thr Leu Met Thr Ile Ala Tyr Val Met
 450 455 460

Ala Ala Ile Cys Ala Leu Phe Met Leu Pro Leu Cys Leu Met Val Cys
465 470 475 480

Gln Trp Arg Cys Leu Arg Cys Leu Arg Gln Gln His Asp Asp Phe Ala
485 490 495

Asp Asp Ile Ser Leu Leu Lys
500

<210> 4
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 4
Ser Glu Val Lys Met Asp Ala Glu Phe Arg
1 5 10

<210> 5
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Peptide

<400> 5
Ser Glu Val Asn Leu Asp Ala Glu Phe Arg
1 5 10

<210> 6
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Peptide

<400> 6
Ser Val Asn Met Ala Glu Gly Asp

1

5

<210> 7

<211> 12

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
Peptide

<400> 7

Lys Gly Val Val Ile Ala Thr Val Ile Val Lys

1

5

10

<210> 8

<211> 4

<212> PRT

<213> Homo sapiens

<400> 8

Asp Thr Ser Gly

1

<210> 9

<211> 8

<212> PRT

<213> Homo sapiens

<400> 9

Leu Val Asn Met Ala Glu Gly Asp

1

5

<210> 10

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 10

ggtaagcatc ccccatgcc ccaacgtc

28

<210> 11
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 11
gacgttgggg ccatggggga tgcttacc 28

<210> 12
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 12
acgttgcttt tgatcgggcc cgaaaacgaa ttgg 34

<210> 13
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 13
ccaattcggt ttcgggcccg atcaaagaca acg 33

<210> 14
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 14
ccatcctaatac gactcact atagggc 27

<210> 15
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 15
actcactata gggctcgagc ggc

23

<210> 16
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 16
cttttgagca agttcagcct ggtaa

26

<210> 17
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 17
gagggtggcct atgagtatctt cttccagggt a

31

<210> 18
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 18
tggcgacgac tcctggagcc cg

22

<210> 19
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 19
tgacaccaga ccaactggta atgg

24

<210> 20
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 20
catatggcgg gagtgtgtgc tgcccac

27

<210> 21
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 21
ggatcctcac ttcagcaggg agatgtcatc agcaaagt

38

<210> 22
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Oxidised
Insulin B-chain

<220>
<223> Xaa at site 3 represents cysteic acid

<400> 22
His Leu Xaa Gly Ser His Leu Val
1 5

<210> 23
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Oxidized
Insulin B-chain

<220>
<223> Xaa at site 1 represents cysteic acid

<400> 23
Xaa Gly Glu Arg Gly Phe Phe Tyr
1 5

<210> 24
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Peptide

<400> 24
Val Gly Ser Gly Val
1 5

<210> 25
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Peptide

<400> 25
Val Gly Ser Gly Val Leu Leu

1

5

<210> 26

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
Peptide

<400> 26

Gly Val Leu Leu Ser Arg Lys

1

5

<210> 27

<211> 7

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Inhibitors

<400> 27

Val Asn Leu Ala Ala Glu Phe

1

5

<210> 28

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Inhibitors

<400> 28

Glu Val Asn Leu Ala Ala Glu Phe

1

5

<210> 29

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Peptide

<400> 29

Asn Leu Ala Ala

1

<210> 30

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic Peptide

<400> 30

Val Gly Ser Gly Val Leu Leu Ser Arg Lys

1

5

10

<210> 31

<211> 326

<212> PRT

<213> Homo sapiens

<220>

<223> Amino acids 2-5, 6-9, 13-20, 25-32, 65-67, 69-74, 79-87, 89-91, 99-106, 119-122, 150-154, 164-167, 180-183, 191-194, 196-199, 201-204, 210-214, 221-223, 258-262, 265-269, and 275-278 are Beta Strands

<220>

<223> Amino acids 281-284, 286-288, 298-301, 310-315, and 319-324 are Beta strands

<220>

<223> Amino acids 48-51, 111-114, 136-142, 225-234, 249-254, 271-274, and 303-306 are Helices

<220>

<223> Amino acids 12-13, 30, 32, 34-35, 73-77, 111, 117, 120, 189, 213, 215, 217-220, 287, 289, 291, 298, and 300 are residues in contact with pepstatin.

<220>

<223> Pepsin

<400> 31

Val Asp Glu Gln Pro Leu Glu Asn Tyr Leu Asp Met Glu Tyr Phe Gly
 1 5 10 15
 Thr Ile Gly Ile Gly Thr Pro Ala Gln Asp Phe Thr Val Val Phe Asp
 20 25 30
 Thr Gly Ser Ser Asn Leu Trp Val Pro Ser Val Tyr Cys Ser Ser Leu
 35 40 45
 Ala Cys Thr Asn His Asn Arg Phe Asn Pro Glu Asp Ser Ser Thr Tyr
 50 55 60
 Gln Ser Thr Ser Glu Thr Val Ser Ile Thr Tyr Gly Thr Gly Ser Met
 65 70 75 80
 Thr Gly Ile Leu Gly Tyr Asp Thr Val Gln Val Gly Gly Ile Ser Asp
 85 90 95
 Thr Asn Gln Ile Phe Gly Leu Ser Glu Thr Glu Pro Gly Ser Phe Leu
 100 105 110
 Tyr Tyr Ala Pro Phe Asp Gly Ile Leu Gly Leu Ala Tyr Pro Ser Ile
 115 120 125
 Ser Ser Ser Gly Ala Thr Pro Val Phe Asp Asn Ile Trp Asn Gln Gly
 130 135 140
 Leu Val Ser Gln Asp Leu Phe Ser Val Tyr Leu Ser Ala Asp Asp Gln
 145 150 155 160
 Ser Gly Ser Val Val Ile Phe Gly Gly Ile Asp Ser Ser Tyr Tyr Thr
 165 170 175
 Gly Ser Leu Asn Trp Val Pro Val Thr Val Glu Gly Tyr Trp Gln Ile
 180 185 190
 Thr Val Asp Ser Ile Thr Met Asn Gly Glu Ala Ile Ala Cys Ala Glu
 195 200 205
 Gly Cys Gln Ala Ile Val Asp Thr Gly Thr Ser Leu Leu Thr Gly Pro
 210 215 220
 Thr Ser Pro Ile Ala Asn Ile Gln Ser Asp Ile Gly Ala Ser Glu Asn
 225 230 235 240

Ser Asp Gly Asp Met Val Val Ser Cys Ser Ala Ile Ser Ser Leu Pro
245 250 255

Asp Ile Val Phe Thr Ile Asn Gly Val Gln Tyr Pro Val Pro Pro Ser
260 265 270

Ala Tyr Ile Leu Gln Ser Glu Gly Ser Cys Ile Ser Gly Phe Gln Gly
275 280 285

Met Asn Leu Pro Thr Glu Ser Gly Glu Leu Trp Ile Leu Gly Asp Val
290 295 300

Phe Ile Arg Gln Tyr Phe Thr Val Phe Asp Arg Ala Asn Asn Gln Val
305 310 315 320

Gly Leu Ala Pro Val Ala
325

Reviewed by: PLW on 3/26/01

Patent#/author: WO 01/00663

Keywords Memapsin 2
Alzheimers

inhibitors

Review (if necessary)

Data:

- Prep of catalytically active recomb memapsin
- Refolding procedure
- Localization
- ~~class~~ Substrate cleavage conditions
- Design of substrate analogs for memapsin 2
- expression of memapsin in cells
- Design of dipeptide ~~isotere~~ inhibitors
- Memapsin crystal structure.

Claims to:

- 1) Purified recombinant active memapsin 2
- 2) Method for producing active memapsin
- 3) Method for isolating inhibitors of cleavage by memapsin
- 4) Method for obtaining inhibitors based on crystal structure
- 5) Inhibitor database.
- 6) Treating/preventing Alzheimers

Perceived significance:

Irrelevant / Somewhat significant / Moderately significant / Highly significant

1 2 3 4 5 6 7 8 9 10

Reviewed by: KMK on 3/27/01

Patent#/author: _____

Keywords memapsin 2

substrate specificity

transition state isoteres

Review (if necessary)

Peptide inhibitors designed with a transition state isotere

PHARMACIA
UPJOHN

REVIEWED TWICE

HIGH

RELEVANCE

Perceived significance:

Irrelevant / Somewhat significant

1 2 3 4